DRAFT RESEARCH STUDY REPORT

A SINGLE-CENTER, PILOT EXPLORATORY STUDY TO EVALUATE STUDY DESIGN, STUDY LOGISTICS, AND BIOANALYTICAL METHODS TO MEASURE SMOKE EXPOSURE IN ADULT SMOKERS OF 3.0 TO 6.9 MG FTC TAR DELIVERY CIGARETTES AS COMPARED TO ADULT NON-SMOKERS (PILOT TOTAL EXPOSURE STUDY)

PROTOCOL NUMBER:	PM-8450
RESEARCH STUDY REPORT NO.	1148
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DATE STUDY STARTED:	01 May 2001
DATE STUDY COMPLETED:	18 June 2001
DATE OF REPORT:	24 July 2002

1.1 Authors' Signatures

In my opinion this report is a true and accurate reflection of the original study data.

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Principal Investigator
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Date

Rachid Chaouki, MS
Statistician
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1.2 Quality Assurance Statement

A SINGLE-CENTER, PILOT EXPLORATORY STUDY TO EVALUATE STUDY DESIGN, STUDY LOGISTICS, AND BIOANALYTICAL METHODS TO MEASURE SMOKE EXPOSURE IN ADULT SMOKERS OF 3.0 TO 6.9 MG FTC TAR DELIVERY CIGARETTES AS COMPARED TO ADULT NON-SMOKERS (PILOT TOTAL EXPOSURE STUDY)

The Quality Assurance Unit of Covance Clinical Research Unit Inc. (CRU) has audited this study utilizing the authorized protocol, Standard Operating Procedures (SOPs), and any applicable regulatory agency regulations. The Quality Assurance Unit conducted the following inspections and has submitted written reports of these inspections to Principal Investigator. Written status reports of inspections and findings are submitted to Management according to Covance CRU SOPs.

Date of Inspection (MM/DD/YYYY)	Type of Inspection	Date distributed or issued to Covance CRU Principal Investigator (MM/DD/YYYY)

NOTE: Type of inspection and dates will be entered at the time of the Final Report.

Cindy Kreuger, CCRC
Supervisor, Quality Assurance Unit
Covance CRU

Date

2. SYNOPSIS

NAME OF SPONSOR/COMPANY:	Philip Morris U.S.A. 4201 Commerce Road Gate C, Door 17 Richmond, Virginia 23234
STUDY TITLE:	A Single-Center, Pilot Exploratory Study to Evaluate Study Design, Study Logistics, and Bioanalytical Methods to Measure Smoke Exposure in Adult Smokers of 3.0 to 6.9 mg FTC Tar Delivery Cigarettes as Compared to Adult Non-Smokers (Pilot Total Exposure Study)
PROTOCOL NO.:	PM-8450
INVESTIGATORS/ INSTITUTIONS:	Principal Investigator: Russell M. Dixon, MD, Covance CRU Sponsor Representative: Roger Walk, PhD, D.A.B.T., Philip Morris U.S.A. Statistician: Rachid Chaouki, MS, Covance CRU
PUBLICATIONS:	None as of the time of this report.
PERIOD OF TRIAL:	01 May 2001 to 18 June 2001

STUDY OBJECTIVES:	• To establish the validity of the design concepts (e.g., sample collection, feasibility, and precision of analytical methods for biomarkers, sample handling and stability, data acquisition by Questionnaire) to be used in a subsequent large study of adult smokers representing all tar and nicotine categories in the U.S. market.
	• In particular, the study should determine:
	  • The intra- and inter-individual variability of each estimate of biomarkers of exposure and biomarkers of effect.
	  • If the selected biomarkers of exposure and effect can differentiate between adult smokers of 3.0 to 6.9 mg tar (FTC) cigarettes and adult non-smokers.

STUDY DESIGN:	This study employed a single-center, out-patient visit design. It was an exploratory observational study with 2 parallel groups (adult smokers and adult non-smokers) and 2 subgroups (male and female) within each group.
STUDY POPULATION:	The initial study population consisted of 140 healthy adult male and female subjects, 21 years of age or older. The population included a distribution of 72 smokers (distributed as 33 adult male smokers and 39 adult female smokers) and 68 non-smokers (distributed as 32 adult non-smoking males and 36 adult non-smoking females). Subject smoking status was defined by cigarette consumption of a minimum of 1 manufactured cigarette per day over the last 12-month period.

2. SYNOPSIS

VISIT SCHEDULE:	The subjects reported to the study site for a Screening visit within 28 days prior to entry into the actual study. After Enrollment, the subjects visited the study site 4 times (i.e., once during each Week 1, 2, 3, and 6). The subjects received the necessary supplies at each visit to collect the needed samples/information for the next scheduled visit. The visits were scheduled such that the blood samples for analysis of biomarkers were conducted on 2 of their leisure days and 2 of their non-leisure days. The 24-hour urine collections were conducted on 2 non-leisure days and 1 leisure day.
SAFETY EVALUATIONS/ SAMPLE COLLECTION PROCEDURES:	Complete laboratory evaluations (chemistry [fasted for a minimum of 6 hours], hematology [CBC], and complete urinalysis [UA]) were collected at Screening and at Enrollment. A urine drug screen was performed for all subjects at Enrollment. For all female subjects, a urine sample was collected at each site visit for pregnancy testing at the research site. At the Screening visit, each subject underwent pulmonary function tests to determine the FVC and FEV_1. If the subject's level was less than 75% of the expected value, the subject was not allowed in the study. A 12-lead ECG was obtained at Screening for exclusionary purposes only. Vital signs (including oral temperature, respirations, and automated seated blood pressure and pulse) were obtained at Screening, Enrollment, and at each site visit. A physical examination was performed at Enrollment. Subjects were asked a non-leading "How Do You Feel?" question at each post-Screening study site visit. Any change in the subject's health status was recorded as an intercurrent illness. This inquiry also determined any concomitant medications that the subjects were using.
BIOMARKER SAMPLE COLLECTION PROCEDURES:	**Exhalate:** Samples of subject exhalate for determination of acetonitrile were collected at site visits during Weeks 1, 3, and 6. Levels of carbon monoxide in exhalate were determined at site visits during Weeks 1, 2, 3, and 6. The collection/determination times were before any other procedures and within 30 minutes of arrival at the study site. **Blood:** Blood samples for determination of acetonitrile and carboxyhemoglobin in blood were collected at site visits during Weeks 1, 2, 3, and 6. Blood samples for determination of hemoglobin adducts in RBCs were collected at site visits during Weeks 1 and 6. Blood samples for determinations of malondialdehyde and fibrinogen in plasma, and HDL- and LDL-cholesterol, and C-reactive protein in serum were collected at site visits during Weeks 1, 3, and 6. Collection of blood samples were after the exhalate collections and within 30 minutes of arrival at the study site. **Urine:** Subjects were instructed on how and when to collect urine samples for the determinations of 11-dehydrothromboxane-B_2, 8-epi-prostaglandin-$F_{2\alpha}$, malondialdehyde, nicotine and nicotine metabolites, and creatinine during Weeks 1, 2, and 3. The urine samples collected on Weeks 1 and 3 were also used for the determination of NNAL and NNAL-glucuronide.

2. SYNOPSIS

BIOMARKER SAMPLE COLLECTION PROCEDURES:	**Urine (Continued):** At Enrollment, a urine sample was collected 4 to 5 hours after a caffeine challenge to determine the CYP1A2 and NAT2 phenotype of the subjects. **Sputum:** All subjects were given a separate collection container for at-home collection of spontaneous sputum on the day of a scheduled visit to the research site. Additionally, an attempt was made at each site visit to collect spontaneous sputum samples. **Diary:** Each subject was asked to keep a diary of exposure to other people's tobacco smoke and/or smoking consumption from the time of 48 hours prior to the urine collection until the end of the 24-hour collection interval (i.e., until they arrived at the study site). **Questionnaire/Survey:** During the Enrollment visit and again at Week 6, a full Questionnaire was administered by a trained interviewer during which the subjects were asked about demographics, chemical exposures (i.e., occupational, hobbies, household, solvents), exposure to other people's tobacco smoke, home heating/ventilation systems, dietary characteristics, use of non-tobacco nicotine products, and physical activity. Smoking behaviors/consumption for smokers were also queried. At each site visit during Weeks 1, 2, 3, and 6, the subjects were asked questions (e.g., Weekly Survey) related to the smoking behaviors/ consumption and exposure to other people's tobacco smoke during the 3 days prior to the site visit.
BIOMARKER STATISTICAL METHODOLOGY:	This pilot study was an observational study which examined the validity of the acquisition of data by Questionnaire and diary for use in interpreting data obtained on various biomarkers. The study determined the intra- and inter-individual variability of each estimate of smoke constituent uptake in adult smokers of cigarettes in the 3.0 to 6.9 mg tar range and of non-smokers. Biomarkers of exposure and biomarkers of effect were compared between smokers and non-smokers, between male smokers and male non-smokers, and between female smokers and female non-smokers. Comparisons were also made by categorical variables gathered in the Case Report Form, Questionnaires, and Weekly Surveys, whenever possible. Overall as well as by-week comparisons were performed. Statistical comparison between groups was based on the Wilcoxon rank-sum test. Analysis of variance was used to examine whether multiple sample collections or a single sample collection on each subject was required for the Total Exposure Study and to obtain intra-subject variability and inter-subject variability. Exposure-response relationships were evaluated to characterize the relationship between a selected set of biomarkers and estimated daily exposure. Adult smokers' daily exposure was estimated using the FTC-reported TAR, FTC-reported CO, or FTC-reported nicotine value depending on the smoke constituent(s) for a particular biomarker. Variables of possible effect on biomarkers of exposure collected in the CRF, Questionnaire, and Weekly Surveys were the main focus of analyses in exposure-response modeling. A nonlinear regression Gompertz model was used for the mean response.

2. SYNOPSIS

BIOMARKER STATISTICAL METHODOLOGY:	Exposure-response relationships were evaluated to characterize the relationship between biomarkers of effect and the main biomarker of exposure (sum of nicotine and nicotine metabolites). Variables of known possible relationship to biomarkers of effect such as gender, age, and body mass index were forced into the model. Levels of nicotine metabolites (both individually and combined) and 4-aminobiphenyl-hemoglobin adducts were compared between non-smokers exposed to environmental tobacco smoke (ETS) from either spouse at home, other individuals at home, work, vehicles, places, or a combination of these to non-smokers not exposed using the Wilcoxon rank-sum test. In addition, correlation analysis was performed between non-weighted and weighted duration and levels of nicotine metabolites and 4-ABP-Hb adducts. A randomly selected subset of subjects' blood and/or urine samples collected for determination of hemoglobin adducts, sum of nicotine and nicotine metabolites, and NNK metabolites (NNAL and NNAL-glucuronide) were analyzed by 2 different laboratories and statistically analyzed using analysis of variance.
CLINICAL SAFETY STATISTICAL METHODOLOGY:	Results of clinical laboratory evaluations (clinical chemistry, hematology, and urinalysis), physical examinations, vital signs, 12-lead ECGs, intercurrent illnesses, and concomitant medications are listed by subject and summarized using descriptive statistics. No formal statistical analysis was performed on the safety data.
BIOMARKER RESULTS:	Parametric as well as nonparametric analyses showed a statistically significant difference in all the selected biomarkers of exposure between smokers and non-smokers by week and overall. Biomarkers of effect malondialdehyde in blood, HDL-cholesterol, fibrinogen, 11-dehydrothromboxane-B_2, and 8-epi-prostaglandin $F_{2\alpha}$ showed a similar statistical significance, with HDL-cholesterol being the only biomarker with significantly larger values in non-smokers. A single sample collection on each subject would be sufficient to meet the objective of the Total Exposure Study since most of the biomarkers showed no time effect. A high between-subject variability and within-subject variability were observed for most biomarkers of exposure. Analytical %CV showed a low inter-assay variability. Exposure-response modeling for biomarkers of exposure indicated a statistically significant effect of self-exposure on most biomarkers. In general, statistical significance for the difference between smokers and non-smokers seemed to be similar regardless of leisure or non-leisure visits. Evaluation of non-smokers exposed and non-smokers not exposed to ETS showed statistically significant differences for the sum of nicotine and nicotine metabolites, *trans*-3-hydroxycotinine, *trans*-3-hydroxycotinine-*O*-glucuronide, nicotine-*N*-glucuronide, and cotinine-*N*-glucuronide at all sample collection times. The 4-aminobiphenyl-Hb adduct, nicotine, and cotinine showed a significant difference only in one of the visits.

2. SYNOPSIS

BIOMARKER RESULTS: (Continued)	All the selected biomarkers for analyses by the 2 laboratories showed a statistically significant difference between the 2 laboratories with the exception of 4-aminobiphenyl-Hb adduct and nicotine-*N*-glucuronide.
SAFETY RESULTS:	All subjects had clinical laboratory evaluation results that were considered not clinically significant. All intercurrent illnesses reported during the study period were considered to be not clinically significant.
SUMMARY AND RECOMMENDATIONS:	• Problems with looping Questionnaire questions and shortcomings of the electronic submission; recommendation is to review options for Questionnaire format for Total Exposure Study; • Sample collections were not problematic since they were "standard" for a unit that routinely does clinical studies; recommendation is to use research units as much as possible for Total Exposure Study; • Number of subjects that could be processed in a certain timeframe was determined; recommendation is to use research units as much as possible for Total Exposure Study since they are familiar with "timed" events; • Analytical methods are now validated and ready for the Total Exposure Study; recommendation is to further refine assays for the Total Exposure Study; • Most of the biomarkers showed no time effect, recommendation is a single sample collection on each subject for the Total Exposure Study; • High inter-subject variability and intra-subject variability were observed for most biomarkers of exposure while analytical %CV showed a low inter-assay variability; recommendation is to further refine assays and statistical methodology; • Information gathered on multiple formats (i.e., CRF and Questionnaire) created discrepancies; recommendation is to only capture information in 1 source.

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4. ABBREVIATIONS AND DEFINITIONS

@	= at
ABP	= aminobiphenyl
3-ABP	= 3-aminobiphenyl
4-ABP	= 4-aminobiphenyl
Abs	= absolute
Add'I	= additional
Alk	= alkaline
ALQ	= above the limit of quantification
ALT	= alanine aminotransferase
AM	= morning
ANOVA	= analysis of variance
approx	= approximately
AST	= aspartate aminotransferase
atm	= atmosphere
BASO	= basophil
bid	= twice a day
BLQ	= below the limit of quantification
bpm	= beats per minute
BSTFA/TMCS	= bis-(trimethylsilyl)trimethylchlorosilane
BUN	= blood urea nitrogen
C	= continuing
°C	= degress Celsius
CA	= can't answer
CAP	= College of American Pathologists
cap	= capsule/caplet
CBC	= complete blood count
CCLS	= Covance Central Laboratory Services
CCRC	= Certified Clinical Research Coodinator
CCRP	= Certified Clinical Research Professional
CFR	= Code of Federal Regulations
Chem	= chemistry panel
cig	= cigarette
CLIA	= Clinical Laboratory Improvement Act
CO	= carbon monoxide
CRF	= Case Report Form
CRU	= Clinical Research Unit

4. ABBREVIATIONS AND DEFINITIONS

cum	= cumulative
%CV	= percent coefficient of variation
CYP1A2	= microsomal enzyme responsible for a large number of biotransformation reactions
dL	= deciliter
ECG	= electrocardiogram
EENT	= eyes, ears, nose, throat
EOS	= eosinophil
8-epi-$PGF_{2\alpha}$	= 8-epi-prostaglandin-$F_{2\alpha}$
ETS	= environmental tobacco smoke
exp	= exposure
ext.	= extension
FEV_1	= forced expiratory volume in one second
°F	= degrees Fahrenheit
fL	= femtoliter
freq	= frequency
FTC	= Federal Trade Commission
FVC	= forced vital capacity
Fx	= fracture
G	= gravitational force
g	= gram
GC	= gas chromatograph
GCP	= Good Clinical Practices
GED	= General Equivalency Diploma
H	= above the clinical reference range
HA	= headache
Hb	= hemoglobin
HDL	= high density lipoprotein
HDPE	= high density polyethylene
HDYF?	= how do you feel inquiry
HEENT	= head, eyes, ears, nose, throat
Hep	= hepatitis
HIV	= human immunodeficiency virus
Hosp	= hospital
HP	= Hewlett Packard
HPLC	= high pressure liquid chromatograph

4. ABBREVIATIONS AND DEFINITIONS

hr	= hour
ICF	= informed consent form
ICH	= International Committee on Harmonization
in.	= inches
Inc.	= incorporated
IRB	= Institutional Review Board
i.u. (or IU)	= international unit
IV	= intravenous
kg	= kilogram
K_3EDTA	= potassium (as trivalent ion) ethylenediaminetetraacetic acid
L	= liter, or below the clinical reference range
lb	= pound
LC-MS/MS	= liquid chromatography with tandem mass spectrum detectors
LDH	= lactate dehydrogenase
LDL	= low density lipoprotein
LLOQ	= lower limit of quantification
LYMPH	= lymphocyte
Max	= maximum
Min	= minimum
min	= minute
MCH	= mean corpuscular hemoglobin
MCHC	= mean corpuscular hemoglobin concentration
MCV	= mean corpuscular volume
mEq	= milli-equivalents
mg	= milligram
mm/dd/yyyy	= month/day/year
mL	= milliliter
mol	= mole
MONO	= monocyte
MWt	= molecular weight
M	= molar (concentration)
mmHg	= millimeters mercury
m^2	= meters squared
NA	= not applicable
NAT2	= N-acetyltransferase
NCS	= not clinically significant

4. ABBREVIATIONS AND DEFINITIONS

Neg.	= negative
NEUT	= neutrophil
ng	= nanogram
nL	= nanoliter
nmol	= nanomole
NNAL	= 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-gluc	= 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronide
NNK	= 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
No., N	= number
NSR	= normal sinus rhythm
NPD	= nitrogen phosphorus detector
OCC	= occasional
obs	= observation
opth	= opthalmic
OS	= left eye
pct.	= percent
pmol	= picomole
pg	= picogram
P.O.	= post office
PO	= oral
Phos	= phosphatase
PM	= evening
ppm	= parts per million
prn	= as needed
qd	= every day
qhs	= every night
qid	= four times daily
QNS	= quantity not sufficient
RBC	= red blood cell
Rev.	= revision
Ref.	= reference
SD	= standard deviation
SE	= standard error
SGPT	= serum glutamic-pyruvic transaminase
SGOT	= serum glutamic-oxaloacetic transaminase
SO	= self and others

4. ABBREVIATIONS AND DEFINITIONS

SOP	= Standard Operating Procedure
SST	= serum separator tube
Tab	= tablet
Tbspn	= tablespoon
TEA	= thermal energy analyzer
TD	= thermal desorption
tech	= technical
TES	= Total Exposure Study
top	= topical
UA	= urinalysis
UCL	= upper confidence limit
Unk	= unknown
UPC	= Universal Product Code
URI	= upper respiratory infection
U.S.A.	= United States of America
UTI	= urinary tract infection
var	= variance
v/v/v	= volume/volume/volume ratio
voc	= vocational
WVP	= work, vehicle, places
WBC	= white blood cell
μg	= microgram
μmol	= micromole

4. ABBREVIATIONS AND DEFINITIONS

Formulas/Description of Calculated Terms

The following definitions are for values that were calculated using data received from Covance Central Laboratory Services. These calculations were performed by Covance Clinical Research Unit Pharmacometrics Department based upon Sponsor request/direction.

Sum of Nicotine and Nicotine metabolites = (Total Nicotine + Total Cotinine + Total 3-hydroxycotinine)

in nmol/mg Creatinine = ((nmol/mg Creatinine) + (nmol/mg Creatinine) + (nmol/mg Creatinine))
in mg/mg Creatinine = ((nmol/mg Creatinine)+(nmol/mg Creatinine)+(nmol/mg Creatinine))*(1 mol/10^9 nmol)*(162.23 g/1 mol)*(1000 mg/g)
in nmol/24hr = ((nmol/24hr) + (nmol/24hr) + (nmol/24hr))
in mg/24hr = ((nmol/24hr) + (nmol/24hr) + (nmol/24hr))*(1 mol/10^9 nmol)*(162.23 g/1 mol)*(1000 mg/g)
in nmol/L = ((nmol/L) + (nmol/L) + (nmol/L))
in mg/L = ((nmol/L) + (nmol/L) + (nmol/L))*(1 mol/10^9 nmol)*(162.23 g/1 mol)*(1000 mg/g)

Acetonitrile

in nmol/L = (Acetonitrile (ng/mL))*(1 mL/10^3 μL)*(4 μL)*(1 mol/41.05 g)*(1 g/10^9 ng)*(10^9 nmol/ 1 mol)*(1/.650 L)
in nL/L @20 °C and 1 atm = (Acetonitrile (nmol/L))*(1 mol/10^9 nmol)*(24.056 L/1 mol)*(10^9 nL/1 L)

per cigarette calculations

the original value divided by the number of cigarettes reported in Weekly Survey Question 4 (example follows)

Sum of Nicotine and Nicotine metabolites (nmol/mg Creatinine)/cigarette = (nmol/mg Creatinine)/(number of cigarettes reported in Weekly Survey Question 4)

This same procedure was done for all per cigarette calculations.

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5. ETHICS

5.1 Institutional Review Board

The Covance Clinical Research Unit (CRU) Institutional Review Board (IRB) granted final approval of Version 1.1 of Final Protocol No. PM-8450 (dated 17 April 2001) on 23 April 2001.

Amendment No. 1 of Protocol No. PM-8450 (dated 27 April 2001) was approved by the Covance CRU IRB on 27 April 2001. The changes made in the amendment were as follows:

- The urine samples for the phenotyping were clarified to be collected at 4 to 5 hours after the administration of the caffeine caplets.
- The formulation type for the caffeine was updated to caplets and not tablets globally in the document.
- The blood sample collection for C-reactive protein, HDL-cholesterol, LDL-cholesterol, and HIV and hepatitis screens was updated to a single 9-mL serum separator tube instead of a separate tube for the HIV and hepatitis screens.
- The temperature for storage of the spontaneous sputum samples was updated to –20°C.

The Covance CRU IRB granted approval of the Volunteer Information and Consent Form (including Inclusion/Exclusion requirements; Rev. 1, dated 10 April 2001) on 10 April 2001.

The subjects began enrolling into the study on 01 May 2001 and the last subject was released from the study on 18 June 2001. The Covance CRU IRB was notified of study completion on 02 July 2001 and an updated completion notice was filed on 27 August 2001.

A copy of Research Study Protocol No. PM-8450 is presented in Appendix 16.1.

5.2 Ethical Conduct of the Study

All aspects of this study that were carried out by Covance CRU were conducted in accordance with the U.S. Code of Federal Regulations (CFR) governing the Protection of Human Subjects (21 CFR 50), Financial Disclosure by Clinical Investigators (21 CFR 54), Institutional Review Boards (21 CFR 56), Investigational New Drug Application (21 CFR 312, Subpart D), and the Declaration of Helsinki. As such, these sections of U.S. Title 21 CFR, along with the applicable ICH Guidelines, are commonly known as Good Clinical Practices (GCP).

5.3 Subject Information and Consent

Before the Screening site visit procedures began, each potential subject was informed of the nature of the study and was given pertinent information as to the intended purpose of the study. The procedures and possible hazards to which the study subjects would be exposed were explained. The Covance CRU IRB-approved Informed Consent Form was then read and signed by the subject and a witness (i.e., Covance CRU employee). Each subject was provided a copy of the Volunteer Information and Consent Form and all were advised that they were free to withdraw from the study at any time.

6. INVESTIGATORS AND STUDY ADMINISTRATIVE STRUCTURE

Principal Investigator:	Russell M. Dixon, MD Medical Director Covance CRU (608) 283-5680 (Office Telephone No.)
Subinvestigators:	William E. Bridson, MD Steven M. Austin, MD Andrea C. Hillerud, MD Thomas E. Murtaugh, MD Covance CRU
Sample Distribution:	Covance Central Laboratory Services 8211 Scicor Drive Indianapolis, Indiana 46214 (317) 271-1200 (Main Telephone No.) (317) 273-4030 (Main Facsimile No.)

Analytical Laboratories:

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7. INTRODUCTION AND BACKGROUND

7.1 Introduction

The overwhelming medical and scientific consensus is that cigarette smoking causes lung cancer, heart disease, emphysema, and other serious diseases in smokers. Smokers are far more likely to develop serious diseases, like lung cancer, than are non-smokers. Not only is the relationship between specific smoke constituents and these disease states not well understood, the amount of smoke and smoke constituents to which a smoker is exposed is also not well-defined. Studies suggest that machine-derived smoke composition data are not suitable measures of smoker exposure to smoke constituents. To determine the exposure of adult smokers to cigarette smoke in the U.S., a more direct evaluation of the levels of smoke constituents or their metabolites in appropriate biofluids has been proposed. This investigation will include evaluation of biomarkers of exposure and biomarkers of effect[1] in adult smokers who regularly smoke brands of 3.0 to 6.9 mg tar yield, as measured by the Federal Trade Commission (FTC) methods, and published periodically by the Tobacco Institute Testing Laboratory.

Criteria for selection of the biomarkers of exposure for this study were based on National Research Council guidelines for validating markers for environmental tobacco smoke (ETS), with modifications described in a 1997 U.S. Occupational Safety and Health Administration workshop on assessment of workplace exposure to ETS (Benowitz, 1999, Ref. 2). The criteria employed for these selections were:

[1] I.e., biomarkers of biologically effective dose and biomarkers of potential harm.

- Unique or nearly unique to tobacco smoke.
- Representative of particulate and gas phase tobacco smoke.
- Representative of health-relevant tobacco smoke constituents.
- Constituent metabolism understood.
- Concentration reflective of uptake of cigarette smoke constituent(s).
- Sensitive and reliable analytical methods available.
- Sampling to acquire material for analysis only minimally invasive.

With these considerations in mind, smoke constituents and corresponding biomarkers of exposure were selected for analysis in this study. The biomarkers chosen, the corresponding smoke constituent, and the appropriate biofluid matrix are shown in Table 7.1-1.

Table 7.1-1 – Biomarkers of Exposure Chosen for Evaluation

Biomarker	Sample Matrix	Smoke Constituent
Acetonitrile	Exhalate, Blood	Acetonitrile
Carboxyhemoglobin	Blood	Carbon Monoxide
CO	Exhalate	Carbon Monoxide
Hb adducts of 3- and 4-aminobiphenyl (3-ABP-Hb and 4-ABP-Hb)	Blood	3- and 4-aminobiphenyl
Nicotine and 5 metabolites	24-Hour Urine	Nicotine
NNAL and NNAL-glucuronide	24-Hour Urine	NNK

In addition to these biomarkers of exposure, the following biomarkers (Table 7.1-2) of effect were selected for analysis, consistent with published investigations.

Table 7.1-2 – Biomarkers of Effect Chosen for Evaluation

Biomarker	Sample Matrix	Health Effect
HDL-cholesterol, LDL-cholesterol	Blood	Atherosclerosis
11-Dehydrothromboxane-B_2	24-Hour Urine	Platelet activation
Fibrinogen	Blood	Cardiovascular disease
8-Epi-prostaglandin-$F_{2\alpha}$ (8-Epi-$PGF_{2\alpha}$)	24-Hour Urine	Lipid peroxidation
C-reactive protein	Blood	Tissue injury
Malondialdehyde	Blood and Urine	Oxidative stress

For many of the selected biomarkers of exposure, analytical methods were available but required further development or refinement.

Initial data on exposures were required to estimate sample sizes required for the larger study. The study described in this report was designed to assess the feasibility of the proposed larger sample-size investigation by focusing on 1 tar yield segment and a non-smoking control population. The tar yield segment selected (3.0 to 6.9 mg tar/cigarette, as measured by FTC methods) comprises only 11% of U.S. smokers (~80% smoke higher yield cigarettes), and challenged sample recruitment procedures. Measurement of biomarker concentrations in adult smokers of these lower yield cigarettes and the non-smoking control group permitted evaluation of the adequacy of the limits of quantification of the analytical methods.

The procedures employed in this exploratory study were of minimal risk to the participants in the study.

8. STUDY OBJECTIVES

The objectives of this pilot study were:

- To establish the validity of the design concepts (e.g., sample collection, feasibility, and precision of analytical methods for biomarkers, sample handling and stability, data acquisition by Questionnaire) to be used in a subsequent large study of adult smokers representing all tar and nicotine categories in the U.S. market.
- In particular, the study determined:
  - The intra- and inter-individual variability of each estimate of biomarkers of exposure and biomarkers of effect.
  - If the selected biomarkers of exposure and biomarkers of effect can differentiate between smokers of 3.0 to 6.9 mg tar (FTC) cigarettes and non-smokers.

9. INVESTIGATIONAL PLAN

9.1 Overall Study Design and Plan: Description

As a pilot study, the study design measured the smoke constituent uptake of biomarkers and their metabolites in adult smokers of cigarettes in the 3.0 to 6.9 mg tar range and also

in adult non-smokers. The measures of uptake were obtained through data from biosample collections, structured questionnaires, physical examinations, cigarette consumption or smoke exposure diaries, and collections of cigarette butts and packs. The information gained from this pilot study is being used to make decisions about the feasibility of biomarkers, questionnaire content and format, sample size, and data analysis for the full-scale Total Exposure Study (TES).

9.2 Discussion of Study Design

This study was designed as a single-center, out-patient visit, exploratory survey study with 2 parallel groups (adult smokers and adult non-smokers) and 2 subgroups (male and female) within each group. Each subject was to report to the research study site at various times over the course of the 6-week study period. The visits for collection of samples/information during Weeks 1, 2, and 3 were to be scheduled at the end of the 24-hour urine collections. The blood collections during Weeks 1, 2, 3, and 6 were to be scheduled such that each subject had collections on 2 of his/her leisure days and on 2 of his/her non-leisure days (Note that the 24-hour urine collections during Weeks 1, 2, and 3 were to be scheduled so that collections occurred on 2 non-leisure days and 1 leisure day).

Blood, urine, exhalate, and spontaneous sputum samples (if any sputum samples were produced spontaneously) were to be collected for determination of selected biomarkers at various times according to the study flow chart (Table 9.2-1) during the course of the 6-week study period.

Physical examinations, 12-lead ECGs, vital signs, and laboratory evaluations were to be performed at Screening, Enrollment, and/or at specified times during the study as shown in Table 9.2-1.

Table 9.2-1: Study Procedures Flow Chart

Study Procedures	Screen[a]	Enrollment	Week 1*	Week 2*	Week 3*	Week 4*	Week 5*	Week 6*
Medical History	X	X[b]						
Physical Exam		X						
12-Lead ECG	X							
Lung Function Tests[c]	X							
Vital Signs[d]	X	X	X	X	X			X
HDYF? Inquiry[e]		X	X	X	X			X
Concomitant Medications[f]	X	X	X	X	X			X
Weekly Survey[g]			X	X	X			X
Complete Questionnaire[h]		X						X
Blood Sampling[i]								
Acetonitrile (Blood)			X	X	X			X
Carboxyhemoglobin (Blood)			X	X	X			X
Hemoglobin adducts (RBCs)			X					X
HDL-cholesterol and LDL-cholesterol (Serum)			X		X			X
Malondialdehyde (Plasma)			X		X			X
Fibrinogen (Plasma)			X		X			X
C-Reactive Protein (Serum)			X		X			X
Urine Sampling[j]								
Malondialdehyde			X	X	X			
Nicotine and Nicotine Metabolites[k]			X	X	X			
NNAL and NNAL-glucuronide[k]			X		X			
Creatinine			X	X	X			
11-Dehydrothromboxane-B_2[l]			X	X	X			
8-Epi-$PGF_{2\alpha}$[l]			X	X	X			
Exhalate Sampling[m]								
Acetonitrile			X		X			X
Carbon Monoxide			X	X	X			X
Spontaneous Sputum Sampling[n]		X	X	X	X			X
Diary Record[o]			X	X	X			X
Cigarette Collection[p]			X	X	X			X
Cigarette Pack Collection[q]			X	X	X			X
Urine Pregnancy Test[r]	X	X	X	X	X			X
Chem, CBC & UA (fasted)[s]	X	X						
CYP1A2 and NAT2 Phenotyping[t]		X						
Hep & HIV Screen	X	X	X	X	X			X
Urine Drug Screen[u]	X	X						

*Visit schedules were to be made to accommodate the subject's schedule as much as it could. However, visits were to be arranged such that a subject completed the sample (blood) collections on 2 of his/her leisure days and 2 of his/her non-leisure days. The urine collections were to be scheduled such that 2 collections were to be conducted on the subject's non-leisure days and 1 collection was to be conducted on the subject's leisure day. If a subject was unable to visit the site during Weeks 1, 2, or 3, the visit could have been done during Weeks 4 or 5. For the Week 6 visit, there was a 7-day window for the visit (i.e., Week 6 ± 7 days).

[a]Subjects underwent a telephone screen prior to visiting the site for the Screening visit. The telephone screen allowed for the initial eligibility of the potential subject to be determined.

[b]Interim medical history only to determine intercurrent illnesses at time of Enrollment.

[c]Lung function tests consisted of FVC and FEV_1 only and must have been >75% of expected to be included in the study.

[d]Vital signs (including oral temperature, respiratory rate, automated seated blood pressure and pulse) obtained at Screening, Enrollment, and at each site visit where a blood and/or urine sample was obtained.

[e]How Do You Feel? inquiries were performed at each post-Screening vital signs measurement. Any reported findings were recorded as an intercurrent illness/physical finding.

[f]Any medications being taken by a subject and the reason for its use were documented during each site visit.

[g]Weekly surveys were administered at the time the subject reported to the research site during Weeks 1, 2, 3, and 6. These surveys included questions regarding the activities within the last 8 to 10 hours, smoking status and/or exposure, and any changes in the subject's overall status since the last visit.

[h]Full Questionnaires were conducted at the Enrollment visit and at Week 6 to assess smoking behaviors and exposure (household, occupational, and casual [i.e., hobbies]), demographics, dietary characteristics, and overall status of the subjects. Trained interviewers at the study site administered the Questionnaires.

[i]Blood samples were collected for determination of acetonitrile and carboxyhemoglobin in whole blood at site visits during Weeks 1, 2, 3, and 6. Blood samples for the determination of hemoglobin adducts of 3- and 4-ABP in RBCs were collected at site visits during Weeks 1 and 6. Blood samples for determinations of HDL-cholesterol and LDL-cholesterol, malondialdehyde, fibrinogen, and C-reactive protein in plasma or serum were collected at site visits during Weeks 1, 3, and 6.

[j]Subjects were instructed to complete urine collections during the 24-hour interval prior to site visits for Weeks 1, 2, and 3. For each subject, 2 collections were to be scheduled on non-leisure days and 1 collection was to be scheduled on a leisure day. Samples collected during Weeks 1, 2, and 3 were analyzed for malondialdehyde, nicotine and nicotine metabolites, and creatinine. The samples collected during Weeks 1 and 3 were also analyzed for NNAL and NNAL-glucuronide.

[k]Results from these analyses were reported as mass/24-hours, mol/24-hours, mass/mg creatinine, and mol/mg creatinine.

[l]Urine samples for 8-epi-$PGF_{2\alpha}$ and 11-dehydrothromboxane-B_2 were collected as part of the 24-hour urine collections. Additionally, creatinine will also be analyzed in these samples so that they can be reported as mass/mg creatinine and mol/mg creatinine.

[m]Exhalate samples for determination of acetonitrile were collected into special Tedlar® bags at site visits during Weeks 1, 3, and 6. Carbon monoxide in exhalate measurements were performed at the research site during Weeks 1, 2, 3, and 6. Collections/measurements occurred within 30 minutes of arrival at the research site.

[n]Spontaneous sputum samples, if any were produced by the subjects, were to be collected on the morning of site visits for Weeks 1, 2, and 3, and during other site visits (i.e., Enrollment and Week 6) as appropriate.

[o]Subjects were instructed to record smoking consumption and/or exposure to other people's smoke in a diary during a 72-hour interval prior to site visit during Weeks 1, 2, 3, and 6.

[p]Smoking subjects were instructed to collect filters/butts of cigarettes smoked during the interval of the 72-hour diary recording interval during Weeks 1, 2, 3, and 6. The number of butts collected and the number recorded in the diary were compared against each other. Any discrepancies were noted.

[q]Smoking subjects were instructed to collect all packs from which a cigarette was removed during the 72-hour interval prior to site visit (i.e., during diary recording period) during Weeks 1, 2, 3, and 6.

[r]Urine samples were collected from female subjects of child-bearing potential at each site visit for pregnancy testing (pregnancy testing was performed at the research site).

[s]Blood samples (after a minimum 6 hour fast) for chemistry and hematology, and urine sample for urinalysis were collected at Screening and Enrollment.

[t]Urine samples were collected 4 to 5 hours after challenge with caffeine for CYP1A2 and NAT2 phenotyping of subjects.

[u]Urine drug screen did not include alcohol.

This study design was suitable to meet the objectives of the pilot study and to determine the procedure and best design aspects for the TES.

9.3 Selection of Study Population

Subjects were recruited from the research site geographic area using advertising in print and via the internet that had been approved by an IRB as per 21 CFR 56.

Prior to having a potential subject report to the study site, a list of telephone questions was answered by the potential subject. The questions asked during the telephone screen included items pertaining to age, gender, smoking status, and general inclusion/exclusion criteria. A list of sample questions that may have been asked during the telephone screen are included in Appendix A of the protocol.

After a successful telephone screening, the potential subject was invited to the site for a Screening visit within 28 days of Enrollment. Prior to the initiation of any study-specific procedures, the subject was asked to provide verification of age (i.e., a form of government-issued identification with birthdate and photo was obtained from each subject and copied for site records, and the subject was asked to sign a form confirming their age as 21 years or older). The site interviewer verified the subject's age by a thorough review of the identification provided by the subject. After verification of the subject's age, he/she was asked to read and sign a Covance CRU IRB-approved Informed Consent Form (ICF).

After the ICF was signed, the following procedures were performed.

1. A medical history was obtained.
2. A 12-lead ECG was obtained.
3. Vital signs (including oral temperature, respiratory rate, and automated seated blood pressure and pulse) were assessed. Seated blood pressure and pulse was measured after the subject had been seated for at least 5 minutes.
4. Blood samples for laboratory evaluations (including chemistry and hematology) and urine samples for urinalysis and a screen of selected illicit drugs (not including alcohol) were collected. A urine pregnancy test was performed for female subjects.
5. Blood samples for hepatitis and HIV screens were collected.
6. Lung function tests (FVC and FEV_1) were conducted as exclusionary factors.

7. Site personnel performed a Screening interview. The Screening interview consisted mainly of questions related to health/medical history, concomitant medications, tobacco use history, and any intercurrent illnesses/physical findings that may have been of importance before the subject enrolled in the study.

9.3.1 Inclusion Criteria

Subjects who met the following criteria were included in the study:

1. Males and females, in good health, 21 years of age and older.
2. Able to understand and willing to sign an Informed Consent Form.
3. Laboratory evaluations (including chemistry, hematology, and urinalysis) within the reference range for the testing laboratory, unless deemed not clinically significant by the Investigator.
4. Negative urine test for selected illicit drugs (did not include alcohol) at Screening and at Enrollment.
5. Negative urine pregnancy test for female subjects at Screening, Enrollment, and throughout the duration of the study period.
6. Smoking status defined as:

   Adult Smokers: regular consumption of a minimum of 1 manufactured cigarette per day for a minimum of the last 12 months.

   OR

   Adult Non-smokers: no smoking for the last year and/or using any nicotine-containing product such as snuff, chewing tobacco, patches, and/or sprays for 3 months prior to Enrollment.
7. Adult smoking subjects whose cigarettes yielded 3.0 to 6.9 mg tar (FTC method) per cigarette. (The tar content was checked using a list of tar content as supplied by the Sponsor.)

9.3.2 Exclusion Criteria

The following excluded potential subjects from the study:

1. Persons under the age of 21 years.
2. Pregnant or nursing women.
3. Clinical manifestations of significant metabolic, hematological, pulmonary, cardiovascular, gastrointestinal, neurologic, hepatic, renal, urological, or psychiatric disorders.

4. Pulmonary function tests of <75% of the expected normal levels for FVC and FEV_1.
5. Renal insufficiency, as defined by serum creatinine levels of >1.3 mg/dL for females and >1.5 mg/dL for males.
6. Active fever at time of Screening or Enrollment, defined as measured oral temperature greater than 100.2°F.
7. Class III or Class IV congestive heart failure.
8. Class I or Class II congestive heart failure with decompensation in their cardiac status within the past 6 months.
9. Active and symptomatic liver disease or presence of liver enzymes more than 1.5 times the upper limit of normal.
10. Positive HIV or hepatitis result at Screening or Enrollment or at any time during the study duration.
11. Presence of an abnormal ECG at Screening, which, in the Investigator's opinion, was clinically significant.
12. History of drug addiction within 12 months prior to study entry (i.e., prior to Enrollment).
13. Any other condition or prior therapy which, in the opinion of the Investigator, would have made the subject unsuitable for the study.
14. Adult Smokers:
    - Consumption of less than 1 manufactured cigarette of a specified brand per day for a minimum of 12 months.
    - Switched brands during the last 3 months prior to Enrollment.
    - Smoked a brand outside of the specified range of tar delivery (3.0 to 6.9 mg/cigarette) within the last 3 months prior to Enrollment.
    - Used a different brand of cigarettes than their preferred brand at a rate of more than 10% of daily consumption during the last 3 months prior to Enrollment.
    - Used any other nicotine-containing product other than manufactured cigarettes (including roll-your-own cigarettes, bidis, snuff, nicotine inhaler, pipe, cigar, chewing tobacco, nicotine patch, nicotine spray, nicotine lozenge, or nicotine gum) within 3 months prior to Enrollment.
15. Adult Non-smokers:
    - History of smoking within the past 12 months.
    - History of use or used any tobacco- or nicotine-containing product (including cigarette, roll-your-own cigarettes, bidis, snuff, nicotine inhaler, pipe, cigar, chewing, nicotine patch, nicotine spray, nicotine lozenge, or nicotine gum) within 3 months prior to Enrollment.

16. Donation or receipt of whole blood or blood products within 3 months prior to Enrollment, or during the study period.
17. Participation in a clinical study for an investigational drug, device, or biologic within 3 months prior to Enrollment.
18. Any person who was a current or former employee of the tobacco industry, or their first-degree relatives (parent, sibling, child).
19. Any person who was a current employee of the contract research organization (i.e., Covance) or their first-degree relatives.

9.3.3 Removal of Subjects

Subjects were informed that they were free to withdraw from the study at any time and for any reason. The Principal Investigator may have removed a subject from the study if, in the Investigator's opinion, it was not in the best interest of the subject to continue the study, such as, the occurrence of an exclusion criterion which was clinically relevant and may have affected subject safety. Notification of discontinuation was to be made immediately to the Sponsor and Study Monitor. In the case of premature discontinuation of study participation, efforts were made to perform all final study day assessments and the Study Completion page of the CRF was completed. The date the subject was withdrawn from the study and the reason for discontinuation was recorded on the subject's CRF.

9.4 Study Conduct

Subjects were requested to alter their daily routines very minimally in order to be available for site visits and sample collections. The visits were to be scheduled such that any given visit was to occur at the end of the 72-hour collection period of the diary/cigarette butts during Weeks 1, 2, 3, and 6 (corresponded to the end of the 24-hour urine sample collection period). Because of the extended collection of the diary and urine samples, the visits were scheduled no less than 4 days apart.

So as to interrupt regular smoking behaviors as minimally as possible, adult smoking subjects were allowed to smoke in designated areas during the site visits.

After Enrollment, subjects were requested to visit the research site during 4 separate occasions. These visits were to be scheduled, as allowed by the subject's schedule, such that the subject visited the site and underwent the blood collections on 2 of his/her leisure

days and on 2 of his/her non-leisure days. The visits during Weeks 1, 2, and 3 were to be scheduled at the end of the 24-hour urine collection and were to be split so that the subjects were completing the collections of urine on 2 of their non-leisure days and 1 of their leisure days. The Week 6 visit was to be scheduled at the subject's convenience on a leisure day (due to split of Week 1, 2, and 3 visits, the Week 6 visit was expected to be a leisure day visit) However, the visit schedule was modified to fit the subject's schedule and may not have followed this scheme.

If a subject was unable to report to the site during one of the first 3 weeks, a visit may have been rescheduled during Weeks 4 or 5 to make up for the missed visit. The collection of samples was in sequential order even if a week was missed. The actual calendar dates of collection were recorded on the sample labels. The subject may have scheduled the Week 6 visit ±1 week from the "nominal" scheduled timepoint for the final visit (i.e., there was a 14-day window for the final site visit).

Study procedures to be performed at the respective site visits are outlined in Table 9.2-1. At the Enrollment visit, the subject was given the supplies he/she needed to collect the samples for the Week 1 visit. The subjects were given instructions on the collections and the Week 1 visit was scheduled. The same procedures were performed at subsequent visits (i.e., when the subject returned the samples for Week 1, they were given the supplies for collection of the Week 2 samples).

At each site visit, the subjects underwent the following procedures: vital signs measurement, "how do you feel?" (HDYF?) inquiry, assessment of concomitant medications, collection of any spontaneous sputum samples (both those collected at home or those produced at the site), blood sampling (both for selected biomarkers [certain markers were not tested at every visit] and for HIV/hepatitis screens [tested at every visit]), exhalate sampling/testing for respective biomarkers, and a urine pregnancy test for females. At site visits on Weeks 1, 2, and 3, the subjects returned the urine collection containers from the 24-hour collections.

9.4.1 Study Visit Schedule

9.4.1.1 Screening

At the time of the Screening visit, after the potential subject and a witness signed the ICF, the potential subject was given a screening number by which to have his/her samples tracked. The screening number consisted of the letter "S" followed by a 3-digit unique subject identification number.

9.4.1.2 Enrollment

When a subject was approved for the study (i.e., all inclusion and exclusion criteria were evaluated), the subject was assigned a number by Covance CRU staff that consisted of the Sponsor's Project Number followed by a 3-digit unique subject identification number (e.g., Subject Numbers 1148-001 to 1148-135). Assignment of numbers was performed based upon the subject's gender and smoking status as follows:

- Subject Numbers 1148-001 through 1148-035 were adult female smokers
- Subject Numbers 1148-036 through 1148-070 were adult male smokers
- Subject Numbers 1148-071 through 1148-103 were adult female non-smokers
- Subject Numbers 1148-104 through 1148-135 were adult male non-smokers

Assignment of subject numbers was in ascending order within the subgroups. If, for any reason, a subgroup did not have enough subjects to complete the numbering sequence, the remaining number(s) were not to be used.

Additional subjects were to be enrolled in a full subgroup as needed. Additional subjects were to be assigned subject numbers beginning with 1148-500 and continuing in sequential order until all subjects received a unique number. No differentiation between numbers assigned to adult smokers and non-smokers was done for the additional subjects (i.e., the numbers were assigned in ascending order regardless of smoking status and gender).

9.4.2 Concomitant Medications

Any medication taken by a subject during the course of the study and reason for its use was documented during each site visit and in the subject's CRF. There were no restrictions on the use of concomitant medications before or during the study.

9.4.3 Subject Compliance

Subject compliance with the protocol specifications was evaluated at each site visit.

9.4.4 Diet, Fluid, and Activity Control

Since this study had an out-patient visit design, no diet, fluid, or activity control was incorporated. The subjects were instructed to continue their normal daily activities during the study period.

9.5 Biomarker and Safety Variables

The following sample collections were conducted at various times during the study. The listed biomarkers were assessed at various timepoints throughout the study period to determine the extent of exposure and uptake of smoke constituents. Safety variables were assessed for informational purposes only (NOTE: Some "safety" parameters were also used as biomarkers of effect).

9.5.1 Biomarker/Information Measurements

Biomarkers were collected through blood, plasma, serum, urine, and exhalate sampling. The samples were analyzed by various validated methods at the analytical laboratories.

9.5.1.1 Sample Collections for Biomarker Analysis

Blood samples were collected by site phlebotomists in collection tubes as outlined in Table 9.5.1.1-1.

Table 9.5.1.1-1 Blood/Plasma/Serum Biomarkers

Biomarker	Sample Matrix/Tube Volume/Type	Collection Schedule
Acetonitrile	Whole Blood in a 6 mL K_3EDTA tube	Weeks 1, 2, 3, and 6
Carboxyhemoglobin	Whole Blood in a 3 mL K_3EDTA tube	Weeks 1, 2, 3, and 6
3-ABP-Hb and 4-ABP-Hb	RBCs from Whole Blood collected in a 10 mL K_3EDTA tube	Weeks 1 and 6
Malondialdehyde	Plasma from 10 mL of blood collected in a K_3EDTA tube	Weeks 1, 3, and 6
HDL-cholesterol, LDL-cholesterol, C-reactive protein	Serum from a 5 mL SST tube	Weeks 1, 3, and 6
Fibrinogen	Plasma from a 2.7 mL Citrate tube	Weeks 1, 3, and 6

The blood samples were processed, as appropriate, at the research site, including the washing of the RBCs for the hemoglobin adducts. The resulting matrices were shipped to Covance Central Laboratory Services (CCLS) for distribution to the appropriate analytical laboratories.

A total of 3 urine collections were performed by the subjects outside of the research site, each over a 24-hour interval, for determination of the biomarkers listed in Table 9.5.1.1-2.

Table 9.5.1.1-2 24-Hour Urine Biomarkers

Biomarker	Number of Aliquots/ Aliquot Volume	Collection Schedule
Malondialdehyde	4 aliquots of 4 mL each	Weeks 1, 2, and 3
Nicotine and Nicotine Metabolites	4 aliquots of 4 mL each	Weeks 1, 2, and 3
NNAL and NNAL-glucuronide	7 aliquots of approximately 100 mL each	Weeks 1 and 3
11-Dehydrothromboxane-B_2	2 aliquots of 5 mL each	Weeks 1, 2, and 3
8-epi-$PGF_{2\alpha}$	2 aliquots of 5 mL each	Weeks 1, 2, and 3
Urine creatinine (24-hour)	1 aliquot of 10 mL	Weeks 1, 2, and 3

The subjects were each given a urine collection device, several large urine collection containers, and a cooler-type storage unit (equipped with ice packs/cooling packs) prior to their scheduled collection period. The subjects were instructed in the collection of the urine samples: they were instructed to collect each void into the urine collection device and that the void should be added to the large collection containers and kept cool. When the subject returned the urine collection for a specified period, the urine was processed at the research site and the aliquots were shipped to CCLS for distribution to the appropriate analytical laboratory.

Exhalate samples for determination of acetonitrile were collected into Tedlar® bags at Weeks 1, 3, and 6 for each subject. The contents of the bags were processed to transfer the exhaled air onto thermal desorption tubes using sampling pumps. The tubes were shipped to CCLS for distribution to the appropriate analytical laboratory. Additionally, the levels of carbon monoxide for each subject was determined using a Micro Medical MicroCO Meter at the research site. The display on the instrument was recorded into the subject's CRF.

Subjects were given a sterile container for collection of spontaneous sputum samples on the morning of their site visits. The subjects were instructed to collect any sputum in the morning of the day they were scheduled to visit the research site. Additionally, the subjects were asked when they returned to the site to attempt to collect any spontaneous sputum sample. The samples were processed at the research site and shipped to CCLS for storage and possible further analysis.

Adult smoking subjects were asked to collect all of their cigarette butts and the packs from which they smoked a cigarette during the 24-hour urine collection intervals.

9.5.1.2 Biomarker Variables

The analytical laboratories determined the concentration of each biomarker in the biofluids and/or exhalate using validated methods.

The relationship between certain biomarkers and the exposure of subjects was determined. Refer to Section 9.7 for the methods of statistical analysis planned in the protocol and Section 11.3 for the methods that were used for the actual analysis.

9.5.1.3 Questionnaire/Weekly Survey

During visits at Enrollment and Week 6, the subjects were administered a questionnaire. This tool allowed for the in-depth collection of demographics, exposure to ETS, exposure to other agents that may have affected biomarkers, and lifestyle/health/dietary information.

After collection of blood and/or urine samples, a trained interviewer escorted the subject to an area separate from other procedures being performed. The interviewer was seated at a computer terminal and the subject was placed such that he/she could not see the screen but had face-to-face interaction with the interviewer. Both subject and interviewer had a hard copy of the questionnaire for ease of following the questions and giving/recording answers. Whenever possible, the answers given by the subjects were recorded directly into the computer system by the interviewer.

The interviewer read aloud the questions as they appeared on the computer screen/hard copy. The subjects were instructed to answer to the best of their ability. All efforts were to be made to have the subjects answer all questions; however, if a subject refused to answer any specific question, the refusal was to be noted by the interviewer. If the subject asked for clarification or indicated confusion about the intent of the question, the interviewer was to mark a check box on the computer screen to indicate the subject's confusion. This allowed for an exploration of questions that might need to be examined further before the larger TES.

At each site visit during Weeks 1, 2, and 3, the subjects were asked a series of questions relating to tobacco smoke exposures (household, transportation, and occupational), primary activities within the past 8 to 10 hours, transportation, and smoking consumption for smoking subjects (i.e., they were give the Weekly Survey). Additionally, before collection of any blood/urine/exhalate samples, smoking subjects were asked for the approximate time of their last cigarette.

9.5.1.4 Diary/Cigarette Butt and Cigarette Pack Collection

A diary was given to each subject before the scheduled urine collections during Weeks 1, 2, 3, and 6. Each subject was instructed to record activities relating to exposure to other people's tobacco smoke (non-smoking subjects) and/or smoking consumption (if a smoking subject) during the 72 hours prior to reporting to the research site. Separate diary types (i.e., with separate instructions) were provided to smokers and non-smokers.

The smoking subjects were given a high density polyethylene (HDPE) container into which they were asked to collect their unsmoked cigarette portions (including filter) during the 72-hour period prior to the research site visits during Weeks 1, 2, 3, and 6 (i.e., during the diary recording interval).

The subjects were also given a ziplock bag into which to collect their cigarette packs that were emptied during the 72-hour period prior to the research site visits during Weeks 1, 2, 3, and 6. The packs were to be used for verification of the brand/type of cigarette filters returned to the site, and any discrepancies were to be noted. If the subject reported to the site with a pack that was not empty, a photocopy of the pack was to be obtained. After verification of the brand/type of cigarettes, all packs were to be photocopied and the empty packs were to be destroyed/discarded by the research site (any packs that still contained cigarettes were to be returned to the subject).

The filters collected were counted, stored at ambient temperature until shipped, and sent to the Sponsor's laboratory (INBIFO) for analysis, according to subprotocols. The number of collected cigarette portions and packs were compared to the recorded smoking consumption in the subjects' diary. Any discrepancies between the recorded amount and the number of butts/packs submitted were to be recorded.

9.5.2 Safety Variables

The following safety variables were assessed at various timepoints throughout the study period to ensure that the subjects remained healthy and/or for exclusionary purposes.

9.5.2.1 12-Lead ECGs

A 12-lead ECG was obtained at Screening only as an exclusionary measure.

9.5.2.2 Screening Lung Function Tests

At Screening, all of the subjects performed a lung function test to determine their eligibility for the study. Spirometry measurements were conducted at the research site using a Spirometrics FLOWMATE V PLUS spirometer (Spirometrics Medical Equipment Company). The subjects were instructed in the performance of the tests prior to the measurements being recorded. If necessary, 2 measurements could have been conducted to ensure the subject understood the instructions/instrument. If the subject's results were satisfactory and met the entrance criteria on the initial measurement, the second measurement/test was not necessary. If, however, the subject's results did not meet the entrance criteria on the initial measurement, the subject performed a second test. In order to be eligible for the study, the FVC and FEV_1 measurements had to be >75% of the expected value.

9.5.2.3 Physical Examinations

A physical examination was performed only at Enrollment.

9.5.2.4 Safety Laboratory Evaluations

Blood samples for chemistry determinations were collected using SST tubes (to obtain approximately 2 mL of serum) and blood samples for hematology determinations were collected using a 3-mL draw K_3EDTA tube. Blood samples were collected at Screening and Enrollment for chemistry and hematology analyses.

Blood samples for HIV antibody, Hepatitis B Surface Antigen, and Hepatitis C Antibody screens were collected at each site visit.

Urine samples for urinalysis were obtained at Screening and Enrollment. A urine sample for a drug screen that included assays for selected narcotics and selected illicit drugs was collected at Screening and Enrollment. In order for subjects to continue participating in the study, the urine drug screen at Enrollment had to be negative and the pregnancy test for females had to be negative.

All blood samples for chemistry, hematology, and HIV/hepatitis screens and all urine samples (with exception of pregnancy tests) were analyzed by CCLS.

9.5.2.5 Phenotyping

The phenotyping of subjects for the CYP1A2 and NAT2 activities was performed at the Enrollment visit. The subjects were asked to refrain from consumption of caffeine or methylxanthine-containing products for a minimum of 8 hours prior to reporting to the site. After collection of any blood/urine samples for other analytes/screens, the subjects were each given 200-mg caffeine (No-Doz®, 1 caplet at 200 mg) with 240 mL of water. A urine sample for phenotyping was collected from each subject between 4 and 5 hours after the caffeine administration. A light caffeine-free meal may have been provided to the subjects after the administration of the caffeine dose.

9.5.2.6 Vital Signs

Vital signs (including oral temperature, respiratory rate, and automated seated blood pressure and pulse) were obtained at Screening, at Enrollment, and at each site visit.

9.5.2.7 Urine Pregnancy Test

A urine sample was collected from each female subject at each site visit for pregnancy testing. The test was performed at the research site using a commercially available kit. If a female subject became pregnant during the study, she would have been immediately discontinued from participation in the study, informed of the possible risks to the fetus by the maternal behaviors (i.e., smoking/drinking), and referred to her primary care provider.

9.6 Data Quality Assurance

The following quality control procedures were performed during this research study.

The source documents that were used to collect data were reviewed by Covance CRU personnel. Data from these documents were transcribed into the CRF and a review of transcribed data was performed to verify accuracy of transcription.

Data from the CRFs were entered twice into the CRU data management system tables. Data were compared and discrepancies were resolved until no differences were found on comparison. After data comparisons were completed, an audit was performed on the database. A computer-generated random sample was drawn on the database. Each data point was verified against the CRF. Covance Standard Operating Procedures (March 1999) require that the 99% UCL be lower than 0.0050 (0.50%). Failure to fall below the 99% UCL results in a systematic review of the database.

Data presented in tabular form in the report were reviewed for accuracy. Text was reviewed for content and to ensure a correct representation of study conduct and results.

The Covance CRU Quality Assurance Unit, utilizing the authorized protocol, Standard Operating Procedures, and applicable regulations, audited this study. The Quality Assurance Unit conducted inspections and submitted written reports of these inspections to the Principal Investigator. Written status reports of inspections and findings were submitted to Management, according to Covance CRU SOPs.

A summary of the quality assurance activities/inspections performed for this study is presented in Section 1.2 of this report.

9.7 Planned Statistical Methods (From Protocol)

9.7.1 Statistical and Analytical Plans: General Descriptive Statistics

The data analysis plan involved several strategies to examine the data that were collected. These strategies included:

- Descriptive analyses which described/summarized the study participants.
- Presentation of estimates of biomarkers.
- Modeling of the biomarkers which characterized the relationships between the biomarkers and study variables.

The descriptive analyses provided a demographic profile of the subjects according to smoking status (smokers and non-smokers). Descriptive statistics of the Questionnaire items provided a comparison of the responses provided by smokers and non-smokers.

Estimates of biomarkers were provided for each group (smoker and non-smoker) and by demographic characteristics (e.g., gender, education). In addition to obtaining estimates of the biomarkers, relationships between the biomarkers and estimates of external exposure was examined by comparing the machine-derived estimates (FTC value) and the number of cigarettes smoked. Further detail on each of the analyses is provided in the following sections.

9.7.1.1 Descriptive Presentation of Data

Univariate measures such as the frequency, mean (arithmetic and geometric), standard deviation, standard error, median, quantiles (5^{th} and 95^{th} percentiles), and range were used to describe the study variables and to descriptively examine any relationships that study variables may have had with the outcomes of interest, biomarker measures.

Screening variables that were used only to determine study eligibility were not included in the descriptive presentation. The variables were summarized both statistically and graphically. The descriptive statistics and graphics aided in identifying statistical outliers, in determining the mathematical form of the underlying distribution, and in summarizing the data. All of the descriptive statistics were performed for each collection time separately and for the mean of all the collection times.

The data were examined to identify statistical outliers using graphical displays such as the box plot and stem-and-leaf graphs. If possible, information from the Questionnaire was used to aid in identifying the reason such observations were identified as outliers. The outliers were evaluated carefully and were excluded only if they could be attributed to error and could not be corrected. If an examination of the data failed to produce an explanation for the outlier, other robust evaluation procedures may have been employed to aid in providing estimates that are less sensitive to the occurrence of outliers.

The data were also examined to identify missing data. Whenever missing data occurred, an attempt was made to determine why the data were missing. Missing data may have occurred due to incomplete data collection or nonresponse to a Questionnaire item. Where possible, an effort was made to obtain the missing data. Other options for missing data included imputing the data, carrying forward the last observation, and elimination.

9.7.1.2 Descriptive Statistics for Selected Questionnaire Variables

Overall summary descriptive statistics for the demographic variables of interest for smokers and non-smokers were used to provide a descriptive profile of the study subjects. The number (N) and percentage (%) for each demographic categorical variable for smokers and non-smokers was presented. For the analytical data and continuous variables obtained from the Questionnaire or physical examination, the statistics that were presented for each tar group and overall included number, mean, standard deviation, median, minimum, and maximum.

Comparisons between smokers and non-smokers were made for variables of interest using the t-test, U-test, or the chi-square test, as appropriate.

9.7.1.3 Descriptive Statistics of Biomarkers of Exposure and Effect

Biomarkers of exposure were measured either in urine, blood, and/or exhaled air, and were used to estimate smoke uptake. In particular, for nicotine and NNK, the sum of their respective metabolites was used to provide estimates of smoke uptake. Biomarkers of effect were measured in blood and/or urine.

Separate analyses were performed for each biomarker for smokers and non-smokers. Summary descriptive statistics for each biomarker including mean, median, standard deviation, minimum, and maximum were determined for the smokers and non-smokers and by demographic variables of interest. Graphical displays, such as box plots, were used to examine the distribution of the data values and to identify outliers.

9.7.2 Statistical and Analytical Plans: Biomarkers

The estimates of the biomarkers of exposure and variables such as demographics and environmental factors were compared between the 2 groups (smokers and non-smokers) using an appropriate parametric or non-parametric approach for each collection time separately and for the mean of all collection timepoints.

9.7.2.1 Exposure Response Modeling – Basic Model

Exposure-response relationships were evaluated to characterize the relationship between the biomarkers and estimated daily exposure at each collection time separately or at the first collection time only (or at the collection time with the most complete data). The choice to use a single collection time or all collection times depended on the results of the simultaneous test of the week variable and week by selected covariates interactions (see model in 9.7.3).

With recognition that the actual dose of a cigarette is unknown, the estimated external exposure was approximated using machine-derived data and/or the number of cigarettes smoked during the analysis period. Results from the Massachusetts Benchmark Study (2000) indicate that "...vapor phase smoke constituents are best described by carbon monoxide, while either nicotine or 'tar' describes particulate phase components equally well." The FTC tar or the FTC CO values were used for all other biomarkers with the assumption that all other smoke constituents are either proportional to tar (particulate phase constituents) or to CO (gas phase constituents). The average number of cigarettes smoked daily was to be determined by a weighted average of the number of cigarettes consumed each day that is not a leisure day and the number of cigarettes smoked each day that is a leisure day.

In the first basic approach, no additional explanatory variables or covariates were introduced into the models to describe the exposure-response relationships. The results of the biomarker determinations for the non-smoker group were to be included in the regression analysis to estimate the intercept (baseline) of the models. Quasi-likelihood estimation was used to determine the parameter estimates. If variance heterogeneity

occurred, suitable variance functions were introduced into the regression models. The following nonlinear regression model (Gompertz, asymmetric sigmoid shape) was considered with estimated exposure as variable X and biomarker mean response as μ:

$\mu = a(\exp(-\exp(-b(x-c))))$

If the biomarkers of exposure were found to correlate with estimated external exposure, the relationship between the biomarkers of exposure and effect was determined.

9.7.2.2 Exposure Response Modeling – Model Refinement

Correlation between independent variables was examined to help refine the number of variables. Scatter plots were examined for continuous variables and box plots for categorical variables. Levels of categorical variables were removed if no response to that level was observed. Levels of small counts of responses were combined with other levels when reasonable and appropriate. Levels of categorical variables may have been combined if no difference appeared from the visual inspection of box plots.

Throughout the model-building process, attention was given to identifying observations that did not fit the hypothesized model(s) well, or that had an unusually strong impact on the model parameters or predictive power. Influence diagnostics were examined to identify influential observations. To assist in understanding any influential observations, data from the Questionnaire were reviewed, and observations may have been excluded from the analyses. Regression models were also assessed for adequacy and goodness of fit.

As model building proceeded, preliminary results were shared with the Sponsor, who could request additional analyses in order to accomplish the study objectives.

Variables of possible effect on the biomarkers from the CRFs, Enrollment Questionnaire, and Weekly Survey (Table 9.7.2.2-1), possibly in conjunction with regression trees and a multiple linear regression procedure available in SAS®, were used in refining the model building by deciding on the most important variables and the maximum order of interactions to be included in the model. Variables of primary importance, such as self-exposure (Table 9.7.2.2-2) and gender, were forced into the model building process. The nonlinear model was linearized for input into the multiple linear regression procedure. If possible, the stepwise procedure was used to refine the model and to obtain initial parameter estimates. A 95% confidence interval or ±2 standard errors of the parameter estimates was used as grid values in the nonlinear procedure. With regard to the

Gompertz model, (μ = d + a(exp(-exp(-b(x-c))))), the "d" was initialized to be the average biomarker response of non-smokers, and "a + d" was fixed to be the upper 90% confidence limit based on data available in the literature. The values of "a" and/or "d" may have been fixed in the case of convergence problems.

The variables selected by the regression tree and the multiple linear procedure in SAS® were then used in conjunction with the nonlinear procedure in SAS® to build the predictive model for each biomarker. Variables were entered into the model in a sequential order. To estimate parameters in the nonlinear procedure, the loss statement was used to specify a quasi-likelihood criterion for estimation. At each step, the quasi-likelihood ratio test comparing the quasi-likelihood for the model with more parameters with the reduced model was used to determine each variable's inclusion/exclusion from the model.

In addition, the relationship between biomarkers of effect and biomarkers of exposure, demographic characteristics, and other variables of interest was analyzed using a nonlinear approach as described above. Biomarker measurements from a single timepoint with the most complete data were selected for analyses. If all timepoints had complete data, the first timepoint was used.

Table 9.7.2.2-1 Variables of Possible Effect on the Biomarkers

Source	Variables	
Case Report Form	Age	Body Mass Index
	Systolic Blood Pressure	Diastolic Blood Pressure
	Pulse	AST (SGOT)
	ALT (SGPT)	LDL
	HDL	CYP1A2 Phenotyping
	Triglycerides	Vitamin Use (*Yes or No*)
	NAT2 Phenotyping	
	Concomitant Medications (*Yes or No*)	
Enrollment Questionnaire	Gender	Spanish/Hispanic/Latino
	Race	Marital Status
	Employment	Exposure to Engine Exhaust
	Exposure to Chemicals	Self Exposure (dose)
	Exposure SO (Spouse + Others)	Air Filtration
	Exposure WVP (Workplace + Vehicle + Places)	
	Alcohol Drinks	Physical Activities
	Beer (%)	Length of Exercise
	Wine (%)	Intensity of Exercise
	Liquor (%)	Nutrition
Weekly Survey	Self Exposure (dose)	
	Exposure SO (Spouse + Others)	
	Exposure WVP (Workplace + Vehicle + Places)	

Table 9.7.2.2-2 Variables of Primary Importance

ENROLLMENT QUESTIONNAIRE (PAST 3 MONTHS) **INFORMATION FROM PAST 3 DAYS WILL BE USED FOR THE WEEKLY SURVEY**
Self Exposure = Sum from Day 1 to Day 84 of (Number of Hours at Job 1/ 24 on Day_i*(Number of Cigarettes from Preferred Brand * Amount of Carbon Monoxide + Number of Cigarettes from Alternate Brand * Amount of Carbon Monoxide) + Number of Hours at Job 2 / 24 on Day_i * (Number of Cigarettes from Preferred Brand * Amount of Carbon Monoxide + Number of Cigarettes from Alternate Brand * Amount of Carbon Monoxide) + ... + Number of Hours Not at Work / 24 Day_i * (Number of Cigarettes from Preferred Brand * Amount of Carbon Monoxide + Number of Cigarettes from Alternate Brand * Amount of Carbon Monoxide)) * How Far Down the Cigarette Is Smoked * (1, 2, or 3) Depending on How Deep the Person Smokes. NOTE: Carbon monoxide was used for vapor phase smoke constituents. Nicotine was to be used for nicotine and nicotine metabolites. Tar was used for all other particulate phase components. The percent of the cigarette smoked was adjusted for the percent of carbon monoxide, nicotine, and tar delivered.
Exposure (Spouse) = Number of Cigarettes Spouse Smoked When Together on a Workday * The Duration Exposed to Spouse's Smoke on a Workday * The Number of Workdays per Week * 12 + Number of Cigarettes Spouse Smoked When Together on a NON-Workday * The Duration Exposed to Spouse's Smoke on a NON-Workday * The Number of NON-Workdays per Week * 12.
Exposure (Others) = Sum of Exposure from All Persons Exposed to their Smoke at Home (Number of Cigarettes $Person_i$ Smoked When Together on a Workday * The Duration Exposed to $Person_i$'s Smoke on a Workday * The Number of Workdays per Week * Number of Weeks in the Past 3 Months Exposed to Tobacco on a Workday + Number of Cigarettes $Person_i$ Smoked When Together on a NON-Workday * The Duration Exposed to $Person_i$'s Smoke on a NON-Workday * The Number of Workdays per Week * Number of Weeks in the Past 3 Months Exposed to Tobacco on a NON-Workday).
Exposure (Workplace) = Sum of Exposure from All Jobs (Hours per Day Exposed in Job_i * The Number of Days per Week Exposed in Job_i * The Number of Weeks in Job_i in the Past 3 Months) * (1, 2, or 3) Depending on How Much Smoke There Is.
Exposure (Vehicles) = Sum of Exposure from All Vehicles (Hours per Week Exposed in $Vehicle_i$ * The Number of Weeks in $Vehicle_i$ in the Past 3 Months) * (1, 2, or 3) Depending on How Much Smoke There Is.
Exposure (Places) = Sum of Exposure from All Places (Hours per Week Exposed in $Place_i$ * The Number of Weeks in $Place_i$ in the Past 3 Months) * (1, 2, or 3) Depending on How Much Smoke There Is.

9.7.3 Statistical and Analytical Plans: Intra-Subject and Inter-Subject Variability

This pilot study examined whether multiple sample collections should be obtained for each subject. Depending on the biomarker, there were 2, 3, or 4 sample collection times. A mixed-effects analysis of variance model was used to model the effect over time for each biomarker. Such a model accounted for the within-subject correlated observations. Three correlation structures were to be considered: compound symmetric (assumes a common correlation over time), auto-regressive (based on a single correlation value that diminishes with time), and the unspecified structure. The choice of correlation structure was made based on Akaike's criterion. The model for the mean was parameterized to

include an effect over time, group classification (smoker versus non-tobacco user), important covariates of interest (e.g., demographics, leisure day versus non-leisure day sample identifier, etc.) that may be time-dependent covariates, and time by covariate interactions. The usual F test with Satterthwaite's adjusted degrees of freedom was used to test for important effects. This model facilitated the estimation of intra-subject variability and inter-subject variability while estimating the effect of important factors of interest.

Multiple correlation coefficients were estimated relating each biomarker with the set of predictor variables. A test for significance of each estimate was based on an F test.

To examine whether multiple sample collections or a single sample collection on each subject was required for the TES, an analysis of variance (ANOVA) model for each biomarker using a repeated measures approach was used, as defined by the following model:

Biomarker = Group + Gender + Week + Group*Gender + Group*Week + Gender*Week + Group*Gender*Week + Random Errors

Where:	Biomarker	= Biomarker
	Group	= Smokers, Non-Smokers
	Week	= Weeks when the biomarker was collected
	Gender	= Male or Female
	Group*Gender	= Group by Gender Interaction
	Group*Week	= Group by Week Interaction
	Gender*Week	= Gender by Week Interaction
	Group*Gender*Week	= Group by Gender by Week Interaction
	Random Errors	= Within- and between-subject errors

Other time-dependent variables were considered for inclusion in the above model.

Akaike's criterion was to be used to decide on the covariance structure among compound symmetric, heterogeneous compound symmetric, auto-regressive, heterogeneous auto-regressive, and the unspecified covariance structures. The above model was used to obtain the intra-subject and inter-subject variability using variability and correlation estimates. The intra-subject and inter-subject variability was obtained for smokers, non-smokers, and overall.

9.7.4 Statistical and Analytical Plans: Supplemental Analyses

Other additional exploratory analyses of interest were performed, as warranted, to accomplish the study objectives. As an example, there exists in the literature reports which claim to have found correlation between subjective ETS exposure estimates and measured biomarker levels. Since biomarker levels were to be measured in the non-smoking group in the Pilot Study, it was of interest to take advantage of the availability of the biomarker data and explore whether any relationship could be established between measured biomarker levels and subjective ETS exposure estimates.

The objective of the analysis was to compare estimated ETS exposure from the Questionnaire response with the measured level of biomarkers of exposure for the non-smoking group. Nicotine metabolites (both individually and combined) and 4-ABP-Hb adducts were chosen as the ETS exposure biomarkers for this study. The choice was based on consideration of (i) the specificity of the biomarkers for exposure to cigarette smoke constituents, (ii) published studies reporting correlation of these biomarkers with estimated ETS exposure, and (iii) sensitivity of analytical methods (originally designed to detect levels in smokers) required to quantify levels of these biomarkers among non-smokers.

Levels of the biomarkers for the ETS exposed and the non-exposed non-smokers were compared using either a parametric test (t-test) or nonparametric test (Wilcoxon rank-sum test). Biomarker levels for the exposed group were further compared with various ETS exposure indices based on the Questionnaire data, e.g., duration/weighted duration, cumulative exposure/average exposure, etc., for individual settings such as home, workplace, etc., and integrated exposure from more than one setting using a regression based approach.

The 10% random subset of blood and/or urine samples of the total number of collected samples for determinations of hemoglobin adducts, nicotine and nicotine metabolites, NNK metabolites, and malondialdehyde analyzed by 2 separate laboratories were to be graphically and statistically compared for each collection time. An ANOVA model for the difference between biomarker values measured by the different laboratories was to be examined using a repeated measures approach as defined by the following model.

Response = Group + Gender + Week + Group*Gender + Group*Week + Gender*Week + Group*Gender*Week + Random Errors

Where:	Response	= Difference in biomarker values measured by different laboratories for the same person
	Group	= Smokers, Non-Smokers
	Week	= Weeks when the biomarker was collected
	Gender	= Male or Female
	Group*Gender	= Group by Gender Interaction
	Group*Week	= Group by Week Interaction
	Gender*Week	= Gender by Week Interaction
	Group*Gender*Week	= Group by Gender by Week Interaction
	Random Errors	= Within- and between-subject errors

Akaike's criterion was to be used to decide on the covariance structure among compound symmetric, heterogeneous compound symmetric, auto-regressive, heterogeneous auto-regressive, and the unspecified covariance structures.

9.7.5 Statistical and Analytical Plans: Safety Variables

Safety variables were summarized using descriptive statistics only and no formal statistical analyses were performed on safety variables that were not considered biomarkers.

9.8 Changes in the Conduct of the Study or Planned Analyses

There were no changes in the conduct of the study; however, there were several modified analyses that were performed after the statistical plan had been approved. A summary of the modified analyses is presented below.

The exposure-response modeling procedure was changed somewhat. The self-exposure calculation was not based on a weighted average of the number of cigarettes smoked on leisure and non-leisure days because this information was not captured in the Questionnaire. The levels for how deep a person inhales were changed from 1, 2, 3 to 1/3, 2/3, and 1 to more accurately reflect the self-exposure level. FTC-reported nicotine values were not used for all nicotine and nicotine metabolites based on information provided by the client. Regression trees (Ref. 9) were not used in the model-building process as this procedure was not sensitive enough to detect significant variables due to the limited data available. Multiple linear regression was not used to refine the model-building process, rather, it was only used to create initial estimates for the nonlinear analysis. Also, gender was not forced into the model-building process, it was removed if not significant. Backward elimination rather than forward selection was chosen as more feasible for model building.

The auto-regressive, heterogeneous auto-regressive, and unspecified structures were not used in the mixed effects modeling since compound symmetry and heterogeneous compound symmetry were thought to adequately model the covariance structure of the steady state biomarker levels.

The analysis of Covance and INBIFO data was modified to test for lab differences regardless of smoking status and gender as requested by the client. Also, malondialdehyde was not analyzed at client request.

10. STUDY SUBJECTS

10.1 Demographics/Dispositions of Subjects

A total of 140 subjects visited the research site at the time of Enrollment. The demographic characteristics of the entire population at Enrollment is presented in Listing 16.5-3 and summarized in Table 10.1-1. A summary of the evaluable subjects' continuous and categorical demographic data is presented in Tables 14.4-1 and 14.4-2, respectively. Additional smoker-related data is presented in Listings 16.6-1 through 16.6-5.

Table 10.1-1 Number of Subjects with Certain Demographic Characteristics

Demographic	Number of Males		Number of Females	
	Smokers	Non-Smokers	Smokers	Non-Smokers
Education				
Completed between 9th and 11th Grade	2	1	1	1
Completed 12th Grade or GED	10	3	8	6
Some tech/voc/trade school, not complete	4	3	5	2
Completed tech/voc/trade school	5	2	8	5
Some College, no degree	6	9	9	9
Completed Undergraduate Bachelor Degree	2	9	6	10
Completed Add'l Post-Graduate Education	4	5	2	3
Marital Status				
Never Been Married	22	17	15	13
Married	9	10	12	15
Divorced	2	4	9	7
Widowed	0	1	1	1
Separated	0	0	2	0
Annual Income				
$19,999 or less	9	9	6	4
$20,000 to $39,999	10	11	13	16
$40,000 to $49,999	8	6	10	9
$50,000 or more	6	6	10	7

Source: All subjects as presented in Listing 16.5-3

Although it appeared that most subjects met all of the inclusion and exclusion criteria at study Enrollment (see Listing 16.5-1), it was determined that several subjects had violated specific criteria. The violations were noted through review of the Questionnaire data and levels of certain biomarkers (specifically non-smoker cotinine values >50 ng/mL). These subjects were excluded from the tables, figures, and analysis and are presented in Listing 16.5-2 with the reason for removal from the analysis. Through these discrepancies, it was concluded that the questions asked by site personnel needed to be more specifically tailored toward the inclusion and exclusion criteria to identify violations prior to the subject completing the study procedures.

The maximum number of evaluable subjects at each timepoint was based on review of the inclusion/exclusion criteria (see Listing 16.5-2) and the availability of a usable response for each subject. The maximum number of evaluable subjects at each timepoint is included in Table 10.1-2. In-text summary tables use only evaluable subjects, unless otherwise noted.

Table 10.1-2 Count of Evaluable Subjects

	Smokers			Non-Smokers		
Timepoint	**Male**	**Female**	**Overall**	**Male**	**Female**	**Overall**
Enrollment	20	33	53	30	35	65
Week 1	18	32	50	30	35	65
Week 2	18	31	49	30	35	65
Week 3	17	29	46	30	35	65
Week 6	14	28	42	30	35	65

A total of 135 subjects completed the study procedures (see Listing 16.5-39) although they were not all included in the analyses after further review of the Questionnaire/Weely Survey data. Five subjects did not complete the entire study period due to positive drug screens or voluntary withdrawal from the study and they are summarized in Table 10.1-3. A complete listing of urine drug screens is presented in Listing 16.5-16. Two subjects had positive screens that correlated to known concomitant medication use and they were allowed to continue on study.

Table 10.1-3 Subjects Removed from the Study Population

Subject Number	Gender/ Smoking Status	Week Withdrawn	Reason for Withdrawal
010	Female/Smoker	After Week 2	Voluntarily withdrew consent, did not show up for Week 3 or Week 6
051	Male/Smoker	Enrollment	Positive drug screen
053	Male/Smoker	Enrollment	Positive drug screen
066	Male/Smoker	Enrollment	Positive drug screen
113	Male/Non-smoker	After Week 2	Voluntarily Withdrew due to Family Emergency

Each subject's reported medical history is presented in Listing 16.5-37.

10.2 Protocol Violations/Deviations

No important deviations related to conduct of the trial (other than those listed below), subject management, or data analysis were recorded.

As described in Section 10.1, several subjects were allowed into the study population that did not meet the entrance criteria as determined by the inclusion and exclusion criteria. This information was not gathered until the time of the Questionnaire analysis.

Subject Number 064 was randomized to the male, smokers group in error. This subject was a non-smoker. Since number 135 was never assigned, this subject was given the 135 number and Subject Number 064 was never used.

Subjects were informed that they should fast for a minimum of 6 hours prior to the Enrollment visit. The subjects were never queried about their fasting status at the time they arrived for the Enrollment visit. They were however, asked about consumption of caffeine and methylxanthine during the previous 8 hours and this answer was recorded.

11. QUESTIONNAIRE/SURVEY/BIOMARKER EVALUATIONS

Listings of the times and dates of collections are included in Listing 16.5-15 (non-smoker diaries) and in Listings 16.5-31 through 34 (blood/plasma, 24-hour urine, exhalate, and sputum, respectively). The leisure/non-leisure visits by subject are listed in Listing 16.5-4. Questionnaire and Weekly Survey data by subject are presented in Listings 16.5-5 through 16.5-14. Descriptive statistics of the biomarkers of exposure are included in the tables presented in Section 14.5, descriptive statistics of the biomarkers of effect are included in the tables presented in Section 14.6, and summaries of the biomarkers (p-values) are included in the tables presented in Section 14.7. Summaries (descriptive statistics and frequencies where applicable) for Questionnaire and Weekly Survey data are presented in the tables included in Section 14.10. Statistical analysis of the biomarkers, dose response modeling, laboratory comparisons, and inter- and intra-subject variability are included in Sections 14.1 through 14.3 and various types of graphical representations of the data are presented in Section 14.11. Listings of individual Questionnaire and Weekly Survey answers by subject are included in Sections 16.5.

11.1 Questionnaire/Survey Evaluations

The Questionnaire and/or Weekly Survey data were used in the analysis of biomarkers and potential relationships. Additionally, a portion of the Questionnaire was modeled after the Fagerström Tolerance Test to determine the degree of nicotine dependency of the smoking subjects. The Fagerström questions (Questions 3.39 through 3.44 on the Smoker's Questionnaire) were rated with the scales in Table 11.1-1.

Table 11.1-1 Fagerström Tolerance Test Classification Scales

Questions	Answers	Points
3.39 How soon after you wake do you smoke your first cigarette?	Within 5 minutes	3
	6 to 30 minutes	2
	More than 30 minutes	1
3.40 Do you find it difficult to refrain from smoking in places where it is forbidden (e.g., in church, at the library, at the movies)?	Yes	2
	No	1
3.41 Which cigarette would you most hate to give up?	The first one in the morning	1
	All others	0
3.42 How many cigarettes per day do you smoke?	10 or less	0
	11 to 20	1
	21 to 30	2
	31 or more	3
3.43 Do you smoke more frequently during the first hours after waking than during the rest of the day?	Yes	1
	No	0
3.44 Do you smoke if you are so ill that you are in bed most of the day?	Yes	1
	No	0
Scoring:		
0 to 2	Very low dependence	
3 to 4	Low dependence	
5	Medium dependence	
6 to 7	High dependence	
8 to 10	Very high dependence	

A listing of the Fagerström results by subject is included in Listing 16.6-5.

11.2 Biomarker Measurements

Analytical methodologies for the determinations of the biomarkers in this study were validated by the analytical laboratory prior their use in this study.

The analytes, the laboratory performing the analysis, and the method used for analysis are presented in Table 11.2-1. Further descriptions of the methods are provided after the table and the respective analytical reports (for those analyses that were performed at a non-clinical analytical laboratory) are provided in Appendix 16.2. Listings of individual biomarker results by subject (for all subjects) are included in Section 16.3 (biomarkers of exposure) and Section 16.4 (biomarkers of effect).

Table 11.2-1 Analytes, Methods, and Published References

Biomarker/Matrix	Analysis Site	Method
Acetonitrile in Blood	Covance Laboratories - Madison	Headspace GC
Acetonitrile in Exhalate	Covance Laboratories - Madison	Thermal Desorption GC-NPD
Carboxyhemoglobin in Blood	Covance Central Laboratories	IL Multi-4 CO-Oximeter (Serial No. 01000875)
3-ABP-Hb and 4-ABP-Hb in RBCs	Covance Laboratories – Harrogate*	GC with negative-ion chemical ionization MS detection
HDL-cholesterol and LDL-cholesterol in Serum	Covance Central Laboratories	Standard CAP and CLIA methods
Malondialdehyde in Blood and Urine	Covance Laboratories – Harrogate	1,3-cyclohexanedione derivatization and HPLC with fluorescence detection.
C-Reactive Protein in Serum	Covance Central Laboratories	Dade Behring Nephelometer
Fibrinogen In Plasma	Covance Central Laboratories	MLA 1600
Nicotine and Metabolites in Urine	Covance Laboratories – Harrogate*	LC-MS/MS
NNK Metabolites in Urine	Covance Laboratories – Madison*	GC with TEA detection
11-Dehydrothromboxane-B_2 in Urine	Covance Laboratories - Harrogate	Microplate immunoassay
8-Epi-$PGF_{2\alpha}$ in Urine	Covance Laboratories - Harrogate	Microplate immunoassay
Carbon Monoxide in Exhalate	Covance Clinical Research Unit	Micro Medical MicroCO Meter

* A sample split was provided to the Sponsor's laboratory (INBIFO) for comparison of results. The results for the laboratory comparisons are described later in this section of the report and are provided in summary tables and graphs in Section 11.1.4.1.

Biomarker: Acetonitrile in Blood

A 3-mL sample aliquot was placed in a 10-mL headspace jar containing 3.0 mL of 0.9% sodium chloride solution. The solution was loaded on the headspace autosampler and agitated at 86°C for 15 minutes prior to injection. The samples were analyzed using the gas chromatograph (GC) parameters outlined in the analytical report. The LLOQ of the method was defined as 30.0 ng/mL. For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

Biomarker: Acetonitrile in Exhalate

The 500 mL Tedlar® bags, equipped with polyvinylchloride mouthpiece/sample inlet valves, were filled by connecting the mouthpiece with an empty tube and adapter to 0.125 inch copper tubing equipped with a Swagelok® nut and Teflon® ferrule system. The tubing was attached to a packed column GC injection port at 250°C through which helium carrier gas flowed at an approximate flow rate of 100 mL/min. Four microliter aliquots of appropriate acetonitrile levels were spiked into the GC injection port, and the bags were allowed to fill for 5 minutes. The bags' contents were transferred to thermal desorption (TD) tubes packed with Tenax/Anasorb GCB1 using an air sampling pump. The tubes were then loaded on the ATD 50 autosampler and analyzed using ATD 50/GC parameters. The LLOQ of the method was defined as 7500 μg/mL. For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

Biomarker: 3-ABP-Hb and 4-ABP-Hb Adducts

Washed RBC samples (approximately 5 mL) were lysed by the addition of ice-cold water (15 mL) followed by phosphate buffer (0.67 M, pH 6.6; 5 mL), prior to ultracentrifugation (38,000g; 30 minutes) to remove the cell debris. Supernatants were transferred into lengths of Visking dialysis tubing (MWt cut-off 12-14,000 Daltons) and dialyzed against 6 changes of water (Milli-Q grade) over a period of 2-3 days. The concentration of hemoglobin in each dialyzate was then determined using Brabkin's method, before hydrolysis of the 3-ABP and 4-ABP hemoglobin adducts with 0.1 M sodium hydroxide (1 hour, room temperature). Liberated ABPs were then concentrated using solid phase extraction (C_{18} reversed phase), eluted with chloroform, and the extracted ABPs taken to dryness using a vacuum centrifuge. ABPs were then derivatized with pentafluoropropionic anhydride in hexane, to form PFP-amines, taken to dryness using a vacuum centrifuge and reconstituted in ethyl acetate prior to analysis using capillary GC-MS. The LLOQ of the method was defined as being equivalent to 0.5 pg/g Hb. For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

For 1 subject (Subject Number 15, Female Non-Smoker), there was an apparent spike in the 3- and 4-ABP-Hb values for Week 6. These values were checked against the raw data and verified that they were correct as presented. After review back to the raw data relayed no discrepancies about the analysis of the sample itself. A subsequent review of the intercurrent illnesses/concomitant medications/exposures and any other factor that may have contributed to this spike was completed. No explanation for the findings was made based on existing data and, therefore, the analysis included this subject's data.

Batches containing reportable data were required to meet acceptance criteria as outlined in the analytical protocol. Due to the complexity of the assay, and the fact that there was no opportunity to perform repeat sample analysis, data were reported from batches that failed to satisfy the protocol acceptance criteria.

Biomarker: Malondialdehyde in Blood (Plasma)

Malondialdehyde is an endogenous compound in human plasma and, therefore, water was used as a replacement calibration matrix within this assay. Malondialdehyde in human plasma/replacement calibrant was subjected to acidification with trichloroacetic acid. Acidified plasma/replacement calibrant was subjected to derivatization with thiobarbituric acid (90 minutes at 95°C plus a further 20 hours refrigerated) prior to injection for analysis using HPLC with fluorescence detection. The LLOQ of the method was defined as 0.4 μmol/L. For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

For the male subjects in this study, there was an apparent "switch" in values for the malondialdehyde in blood between Weeks 1 and 3 as compared to Week 6 (i.e., the values for the smokers were much lower at Week 6 than those seen at the previous 2 timepoints and the non-smokers values were much higher at Week 6 than those seen at the previous 2 timepoints). The analytical laboratory was queried and confirmed that the raw data matched the values that were in the downloaded and analyzed data.

Biomarker: Malondialdehyde in Urine

Similar to the malondialdehyde in plasma method outlined above, water was used as a replacement calibration matrix within this assay. Malondialdehyde in human urine/replacement calibrant was subjected to acidification with trichloroacetic acid. Acidified plasma/replacement calibrant was subjected to derivatization with thiobarbituric acid (90 minutes at 95°C). After cooling, samples were mixed and submitted for analysis using HPLC with fluorescence detection. The LLOQ of the method was defined as 0.4 μmol/L. For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

Biomarker: Nicotine and Nicotine Metabolites in Urine

Nicotine, cotinine (both free and cleaved from their glucuronides) and *trans*-3-hydroxycotinine and their respective deuterated internal standards were extracted from human urine using mixed-mode solid phase extraction (reversed phase/cation exchange). Analytes were eluted with ammonia (5% v/v) in methanol and, following evaporation of the elution solvent, extracts were dissolved in reconstitution solution (10 mM ammonium

acetate, pH native : methanol : triethylamine 800:200:0.1 v/v/v) and injected for analysis using LC-MS/MS. The LLOQ of the method was defined as 1 ng/mL.

Reported values are expressed in terms of "free" and "total" analyte concentrations. Free concentration refers to the levels of non-conjugated analytes (aglycones) nicotine, cotinine, and *trans*-3-hydroxycotinine present in each sample. Total concentration refers to the sum of the free concentration and the concentration of nicotine, cotinine, and trans-3-hydroxycotinine liberated from their respective glucuronides following enzymatic deconjugation.

In the absence of analytical reference standard material for *trans*-3-hydroxycotinine-*O*-glucuronide, reported concentrations for "total" *trans*-3-hydroxycotinine are an estimate only.

For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

Biomarker: NNK Metabolites in Urine

The NNK metabolites and the internal standard were extracted from human urine using ethyl acetate, partitioned to pH 2.0 water, and purified with solid-phase extraction on an Oasis® column. The purified and dried extracts were derivatized with *bis*-(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS) for detection by GC with thermal energy analysis (GC-TEA). The LLOQ of the method was defined as 6 pg/mL. For further information on the assay and individual results, refer to the analytical report, included in its entirety in Appendix 16.2.

Biomarkers Not Analyzed at Analytical Laboratories:

11-dehydrothromboxane-B_2 and 8-epi-$PGF_{2\alpha}$

These analytes were analyzed by the clinical laboratory at Covance Laboratories - Harrogate and there are no formal written reports for these analytes.

HDL- and LDL-cholesterol, Fibrinogen, C-reactive Protein, and Carboxyhemoglobin

These analytes were analyzed by Covance Central Laboratory Services and there are no formal written reports for these analytes.

Carbon Monoxide

The carbon monoxide levels were measured directly at the research site and recorded in each subject's respective CRF.

11.3 Biomarker/Questionnaire Statistical Evaluations

General

Formal statistical analyses were performed and graphics were displayed for a selected set of biomarker units (see Table 11.3-1). Listings and summary tables are displayed for all biomarker units.

Table 11.3-1 Selected List of Biomarkers Used in Formal Statistical Analyses and Graphs

Biomarker	Unit
Acetonitrile in blood	µg/mL
Carboxyhemoglobin	% saturation
3-ABP-Hb adduct	pg/g hemoglobin
4-ABP-Hb adduct	pg/g hemoglobin
Sum of Nicotine and Nicotine metabolites	nmol/mg creatinine
Nicotine	nmol/mg creatinine
Cotinine	nmol/mg creatinine
Trans-3-hydroxycotinine	nmol/mg creatinine
Trans-3-hydroxycotinine-*O*-glucuronide	nmol/mg creatinine
Nicotine-*N*-glucuronide	nmol/mg creatinine
Cotinine-*N*-glucuronide	nmol/mg creatinine
NNAL plus NNAL-gluc	pmol/mg creatinine
NNAL	pmol/mg creatinine
NNAL-*O*-glucuronide	pmol/mg creatinine
Acetonitrile in exhalate	nL/L @20°C and 1 atm
Carbon monoxide	ppm
HDL-cholesterol	mg/dL
LDL-cholesterol	mg/dL
Fibrinogen	mg/dL
C-reactive protein	mg/dL
Malondialdehyde in blood	µmol/L
11-Dehydrothromboxane-B_2	pg/mg creatinine
8-Epi-$PGF_{2\alpha}$	pg/mg creatinine
Malondialdehyde in urine	µmol/mg creatinine

Exposure variables

The calculations used to derive exposure variables are presented in Table 11.3-2.

Table 11.3-2 Calculations of Exposure Variables

Self-exposure	Total number of cigarettes from preferred and alternate brand during either past 3 months prior to Enrollment or past 3 days prior to sample collection times the corresponding FTC reported CO, Nicotine, or Tar Value times the length of cigarette consumed raised to the power of 1.27 times how deep a person inhales (1/3=into the mouth, 2/3=into the throat, 1=into the chest and lungs).
Exposure from Spouse	Total number of cigarettes smoked when together times the duration (hours) during past 3 months prior to Enrollment or past 3 days prior to sample collection.
Exposure from Others	The sum of the total number of cigarettes smoked by each person when together at home times the duration (hours) during past 3 months prior to Enrollment or past 3 days prior to sample collection.
Exposure at workplace	Duration (hours) of exposure during past 3 months prior to Enrollment or past 3 days prior to sample collection times the intensity of smoke (1=light, 2=moderate, 3=heavy).
Exposure in vehicles	Duration (hours) of exposure during past 3 months prior to Enrollment or past 3 days prior to sample collection times the intensity of smoke (1=light, 2=moderate, 3=heavy).
Exposure in places	Duration (hours) of exposure during past 3 months prior to Enrollment or past 3 days prior to sample collection times the intensity of smoke (1=light, 2=moderate, 3=heavy).

The choice of CO, Nicotine, or Tar depended on the biomarker's smoke constituent. The choice of adjustment by 3-month period or 3-day period depended on the biomarker's half-life. Calculations were performed using both 3-month exposure period and 3-day exposure period for biomarkers with unknown half-lives.

Descriptive Statistics and Nonparametric Analyses

Univariate measures such as frequency, arithmetic mean, geometric mean, standard deviation, standard error, quantiles (5^{th} and 95^{th} percentiles), and range were presented for

all biomarkers of exposure and biomarkers of effect. Descriptive statistics were also presented for CRF, Enrollment Questionnaire, and Weekly Survey data. Graphical displays such as frequency plots, scatter plots, box-plots, and interaction plots were presented for biomarkers with selected units of interest and data collected on Week 1 only. Graphical display of trend of biomarkers over time was also presented.

Biomarkers of exposure and biomarkers of effect were compared between smokers and non-smokers, between male smokers and male non-smokers, and between female smokers and female non-smokers. Wherever possible, comparisons were made by categorical variables gathered in the CRF, Questionnaire, and Weekly Survey. Overall as well as by-week comparisons were also performed. Statistical comparison between groups was based on the Wilcoxon rank-sum test.

In addition, for selected biomarkers, biomarker values from blood draws/exhalations taken in the AM were compared with those taken in the PM for smokers and non-smokers.

Outlier data identified through graphical display, listings, or summary tables were reported to the source analyst group. Data were evaluated but no reason for exclusion was found.

Intra-Subject and Inter-Subject Variability and Time Effect

To examine whether multiple sample collections or a single sample collection on each subject was required for the TES, a mixed-effect analysis of variance (ANOVA) (Ref. 3) model for each selected biomarker of exposure and biomarker of effect using a repeated measure approach was used. Four covariance structures were considered: compound symmetry, compound symmetry by smoking status, heterogeneous compound symmetry, and heterogeneous compound symmetry by smoking status. The choice of covariance structure was based on Akaike's criterion (Ref. 4). The model used in the analyses was as follows:

Biomarker = Group + Gender + Week + Group*Gender + Group*Week + Gender*Week
Group*Gender*Week + Random Error.

Where:

Biomarker	=	Biomarker
Group	=	Smoker or Non-Smoker
Week	=	Weeks when the biomarker was collected
Gender	=	Male or Female
Group*Gender	=	Group by Gender Interaction
Group*Week	=	Group by Week Interaction
Gender*Week	=	Gender by Week Interaction
Group*Gender*Week	=	Group by Gender by Week Interaction
Random Error	=	Within-subject covariance structure

To determine if the biomarker data varied by week, a simultaneous test was performed. The full model above was compared to the reduced model containing Group, Gender, and Group by Gender interaction using a likelihood ratio test. Intra-subject and inter-subject variability were obtained by assuming a compound symmetry by smoking status covariance structure.

Several options were exercised to resolve convergence problems. Options used were an increase in the number of iterations, the use of a simpler covariance structure, or the change of a biomarker's unit.

Exposure Response Modeling for Biomarkers of Exposure

Exposure-response relationships were evaluated to characterize the relationship between a selected set of biomarkers (Table 11.3-3) and estimated daily exposure (Table 11.3-2). With recognition that the actual dose of a cigarette is unknown, the estimated external exposure was approximated using machine-derived data and the number of cigarettes smoked during the study period. Results from the Massachusetts Benchmark Study (2000) (Ref. 5) indicated that "...vapor phase smoke constituents are best described by carbon monoxide, while either nicotine or "tar" describes particulate phase components equally well." Adult smokers' daily exposure (self-exposure) was estimated using the FTC reported TAR, FTC reported CO, or FTC reported nicotine value depending on the biomarkers' smoke constituents.

?particulate >

Table 11.3-3 Biomarkers Used in Exposure-Response Modeling

Biomarker	Unit	Half-Lives Assumed (Days)		Phase	Response
Acetonitrile in blood	ng/mL	≤ 3		Gas	Carbon Monoxide
Carboxyhemoglobin	% saturation	≤ 3		Gas	Carbon Monoxide
3-ABP-Hb adduct	pg/g hemoglobin		> 3	Particle	Tar
4-ABP-Hb adduct	pg/g hemoglobin		> 3	Particle	Tar
Sum of Nicotine and Nicotine metabolites	nmol/mg Creatinine	≤ 3	> 3	Particle	Nicotine
Nicotine	nmol/mg Creatinine	≤ 3	> 3	Particle	Tar
Cotinine	nmol/mg Creatinine	≤ 3	> 3	Particle	Tar
Trans-3-hydroxycotinine	nmol/mg Creatinine	≤ 3		Particle	Tar
Trans-3-hydroxycotinine-*O*-glucuronide	nmol/mg Creatinine	≤ 3		Particle	Tar
NNAL plus NNAL-gluc	pmol/mg Creatinine		> 3	Particle	Tar
NNAL	pmol/mg Creatinine		> 3	Particle	Nicotine
NNAL-*O*-glucuronide	pmol/mg Creatinine		> 3	Particle	Nicotine
Nicotine-*N*-glucuronide	nmol/mg Creatinine	≤ 3	> 3	Particle	Nicotine
Cotinine-*N*-glucuronide	nmol/mg Creatinine	≤ 3	> 3	Particle	Nicotine
Carbon monoxide	ppm	≤ 3		Gas	Carbon Monoxide

Source: Table 14.1.3-2.

Acetonitrile in exhalate was not modeled due to severe convergence problems with no resolution. Based on results in the time effect modeling, only Week 1 data were used for analyses. Variables of possible effect on biomarkers of exposure as identified in Table 11.3-4 were the main focus of analyses in exposure-response modeling. Levels of categorical variables were either removed or combined if no or few responses were observed. The following nonlinear regression model (Gompertz, asymmetric sigmoid shape [Ref. 6]) was considered: $\mu = d + a\,(\exp(-\exp(b_0 + b_1x_1 + b_2x_2 + \ldots + b_{24}x_{24})))$. Due to convergence problems 'd' was set to 0 and 'a' was set to the maximum observed data point. X_1 represents self-exposure and x_2 - x_{24} represent variables collected on the CRF, Questionnaire, and Weekly Survey.

Table 11.3-4 Variables Considered in Exposure-Response Modeling

Variable	Source
Self –exposure	Questionnaire/Survey
Age (years)	CRF
Alcohol Use	Questionnaire
Body Mass Index (kg/m^2)	CRF
Diastolic Blood Pressure (mmHg)	CRF
Exercise	Questionnaire
Use of Air Filtration Device	Questionnaire
Type of Heating System At Home	Questionnaire
Exposure to Chemicals and/or Engine Exhaust at the Job	Questionnaire
Job Status	Questionnaire
Marital Status	Questionnaire
NAT2 Phenotyping	Labs
Exposure to Cigarette Smoke From Visitors to Subject's Home	Questionnaire/Survey
Exposure to Cigarette Smoke in Places (Bar, Restaurant, etc.)	Questionnaire/Survey
Pulse (bpm)	CRF
Servings High in Fat (per week)	Questionnaire
Gender	CRF
Exposure to Cigarette Smoke From Spouse at Home	Questionnaire/Survey
Systolic Blood Pressure (mmHg)	CRF
Triglycerides (mmol/L)	Labs
Exposure to Cigarette Smoke in Vehicles	Questionnaire/Survey
Use of Vitamins	CRF
Exposure to Cigarette Smoke at the Work Place	Questionnaire/Survey
CYP1A2 Phenotyping	Labs
Source: Table 14.1.3-1.	

The variable self-exposure was forced into the model building process. The nonlinear model was linearized for input into the multiple linear regression procedure to obtain initial estimates for each parameter. The nonlinear modeling procedure used to refine the full model was as follows:

1. Remove all variables with a Wald (Ref. 7) p-value ≥ 0.5.
2. Test the reduced model to the full model using the quasi-likelihood ratio test (Ref. 8).
3. Remove variables if the quasi-likelihood p-value ≥ 0.15.

4. If the quasi-likelihood p-value < 0.15, put back variables in the model in the order of most significant to least significant based on the Wald p-value until the quasi-likelihood p-value ≥ 0.15.
5. Repeat Steps 1 to 4 for Wald p-values of 0.2 and 0.15.
6. For all remaining variables, add two-way interaction terms with self-exposure in the model with starting values set to 0.
7. Test the additive model to the model with interaction terms using a quasi-likelihood ratio test.
8. Repeat Steps 2 to 4.
9. Randomly select at most 50 combinations of parameter estimates using ±2 or ±1 standard errors of the parameter and check if all models converge to the same location.
10. Obtain quasi-likelihood p-values for all the remaining terms in the model.
11. Remove all the remaining terms with p-values ≥ 0.15 in the order of least significant to most significant until all quasi-likelihood p-values of the remaining terms < 0.15.

During the entire modeling process, initial estimates for a given model were derived from the preceding model. In case of convergence problems, different initial estimates were used ranging anywhere between ± 6 standard errors of the parameter estimates. In some rare cases, more parameters were removed than required by the procedure above to obtain convergence.

Exposure Response Modeling for Biomarkers of Effect

Exposure-response relationships were evaluated to characterize the relationship between biomarkers of effect (LDL-cholesterol, fibrinogen, C-reactive protein, 11-dehydrothromboxane-B_2, and 8-epi-$PGF_{2\alpha}$) and the main biomarker of exposure (sum of nicotine and nicotine metabolites). HDL-cholesterol was not modeled as this biomarker exhibits an inverse trend (i.e., decreases with increasing exposure) compared to all other biomarkers. Malondialdehyde in plasma and urine were not modeled since these biomarkers will not be examined in the TES. Only Week 1 data were used in the analyses.

Variables of known possible relationship to biomarkers of effect such as gender, age, and body mass index were forced into the model. The following nonlinear regression model was considered: $\mu = a\ (\exp(-\exp(b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4)))$ with 'a' set to the

maximum observed data point. The nonlinear model was linearized for input into the multiple linear regression procedure to obtain initial estimates for each parameter. Quasi-likelihood p-values were obtained for each model term. No model refinement was attempted.

Environmental Tobacco Smoke (ETS) Evaluation

Levels of nicotine metabolites (both individually and combined) and 4-ABP-Hb adducts were compared between non-smokers exposed to ETS from either spouse at home and/or other individuals at home and/or work and/or vehicles and/or places to non-smokers not exposed using the Wilcoxon rank-sum test. The ETS exposure calculation was based on 3-day as well as 3-month exposure. Comparison between non-smokers exposed and non-smokers not exposed was done for every single source of exposure, as well as a combination of sources of exposures such as at-home exposure (ETS from at least spouse or other individuals at home) and outside-home exposure (ETS from at least work, vehicles, or places). Comparisons were also made between non-smokers exposed and not exposed to ETS regardless of source of exposure.

In addition, correlation analysis was performed between non-weighted duration and weighted duration (intensity of exposure to ETS: light, moderate, or heavy was used as the weighting factor for work, vehicles, and places while the number of cigarettes smoked was used as the weighting factor for spouse or other individuals at home) and levels of nicotine metabolites and 4-ABP-Hb adducts.

Laboratory Comparison

A random subset of subjects' blood and/or urine samples were collected for determination of hemoglobin adducts, nicotine and nicotine metabolites, and NNK metabolites by both Covance and INBIFO. The model below was used to analyze the difference between biomarker values measured by the different laboratories.

Biomarker = Subject + Week + Subject*Week + Laboratory + Random Error.

Where:

Biomarker	=	Biomarker
Subject	=	Random subject effect
Week	=	Weeks when the biomarker was collected
Subject*Week	=	Random subject by week effect
Laboratory	=	Covance or INBIFO
Random Error	=	Within-subject error

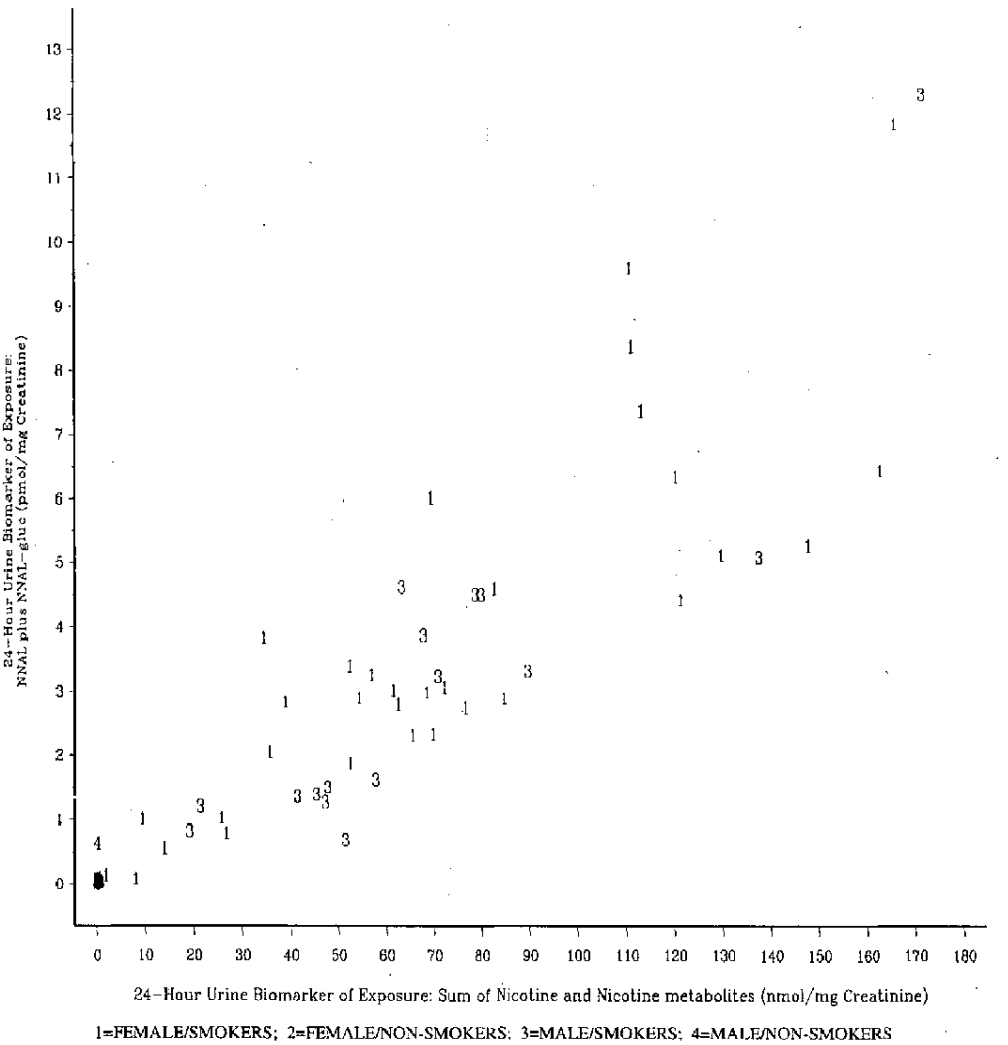
All values below the limit of quantification were removed from this analysis.

All statistical analyses were performed using SAS® version 8.2. Statistical significance was declared at 0.05 significance level.

11.4 Questionnaire/Survey/Biomarker Results and Discussion

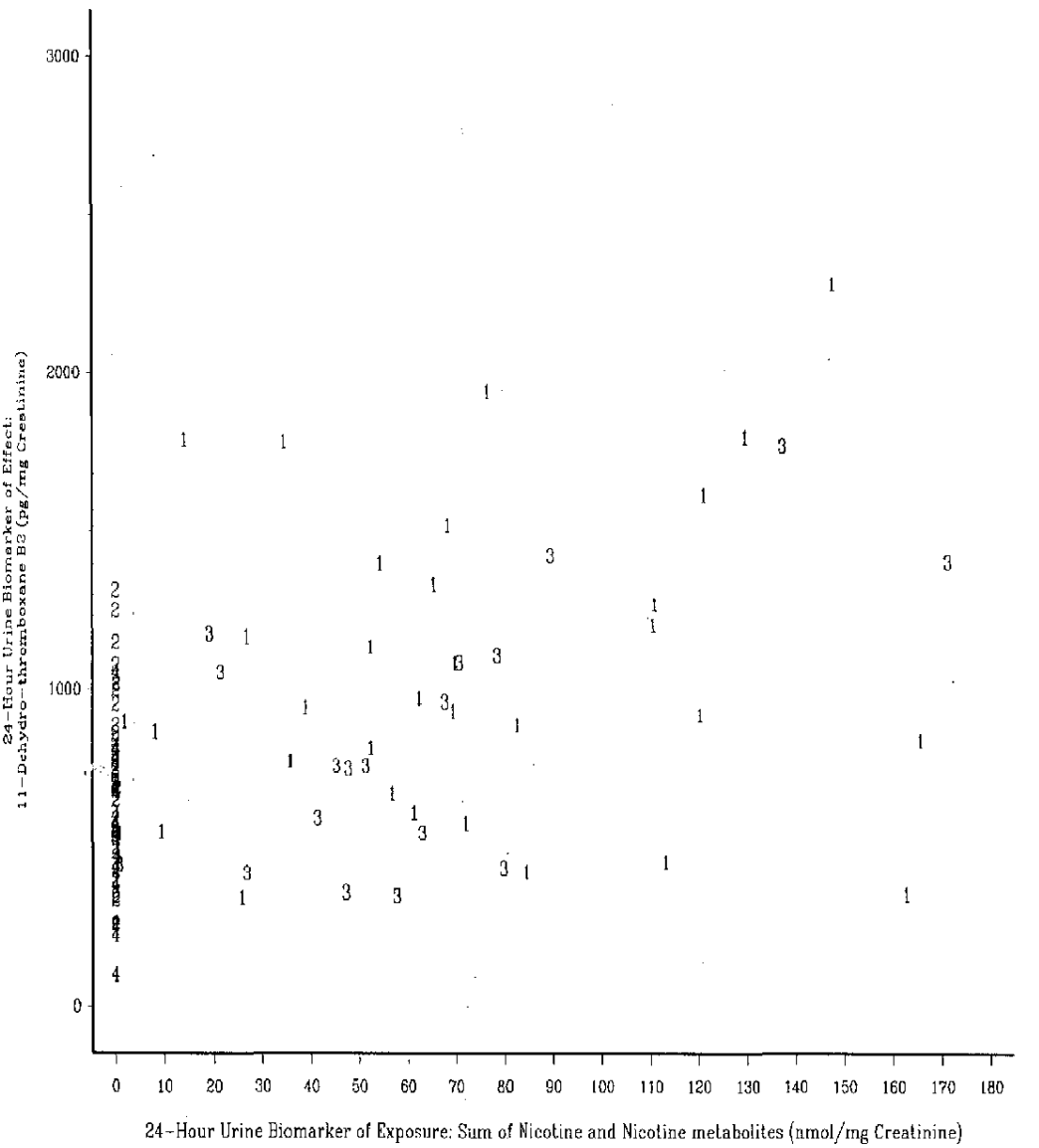
11.4.1 General

Scatter plots, Figures 11.4.1-1 and 11.4.1-2, show the relationship between the Sum of Nicotine and Nicotine metabolites and biomarker of exposure NNAL plus NNAL-glucuronide and between the sum of nicotine and nicotine metabolites and biomarker of effect 11-dehydrothromboxane-B_2, respectively, for Week 1 data. The plots show that as the sum of nicotine and nicotine metabolites increased, the levels of NNAL plus NNAL-glucuronide tended to increase. However, the relationship between the Sum of Nicotine and Nicotine metabolites and 11-dehydrothromboxane-B_2 seemed to be weaker. Similar trends were observed between the sum of nicotine and nicotine metabolites and most biomarkers of exposure and biomarkers of effect across all weeks.



Source: Figure 14.11.5-9

Figure 11.4.1-1 Scatter Plot of Sum of Nicotine and Nicotine Metabolites versus NNAL plus NNAL-glucuronide

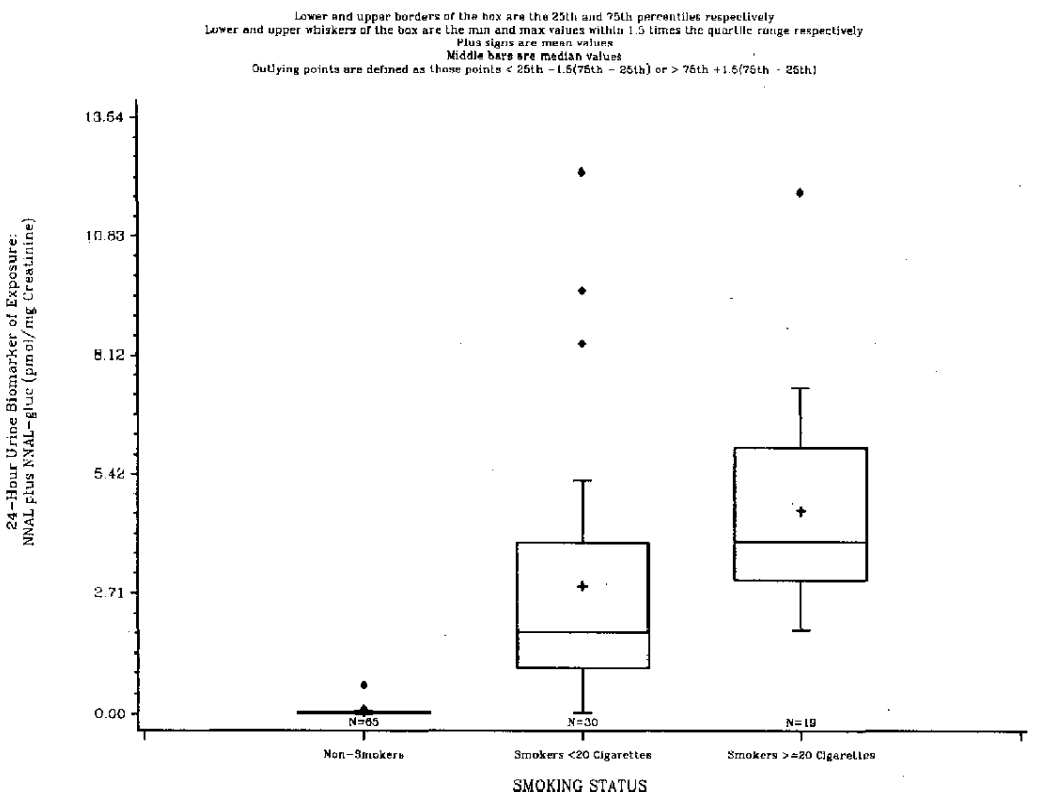


Source: Figure 14.11.5-21

Figure 11.4.1-2 Scatter Plot of Sum of Nicotine and Nicotine Metabolites versus 11-Dehydrothromboxane-B_2

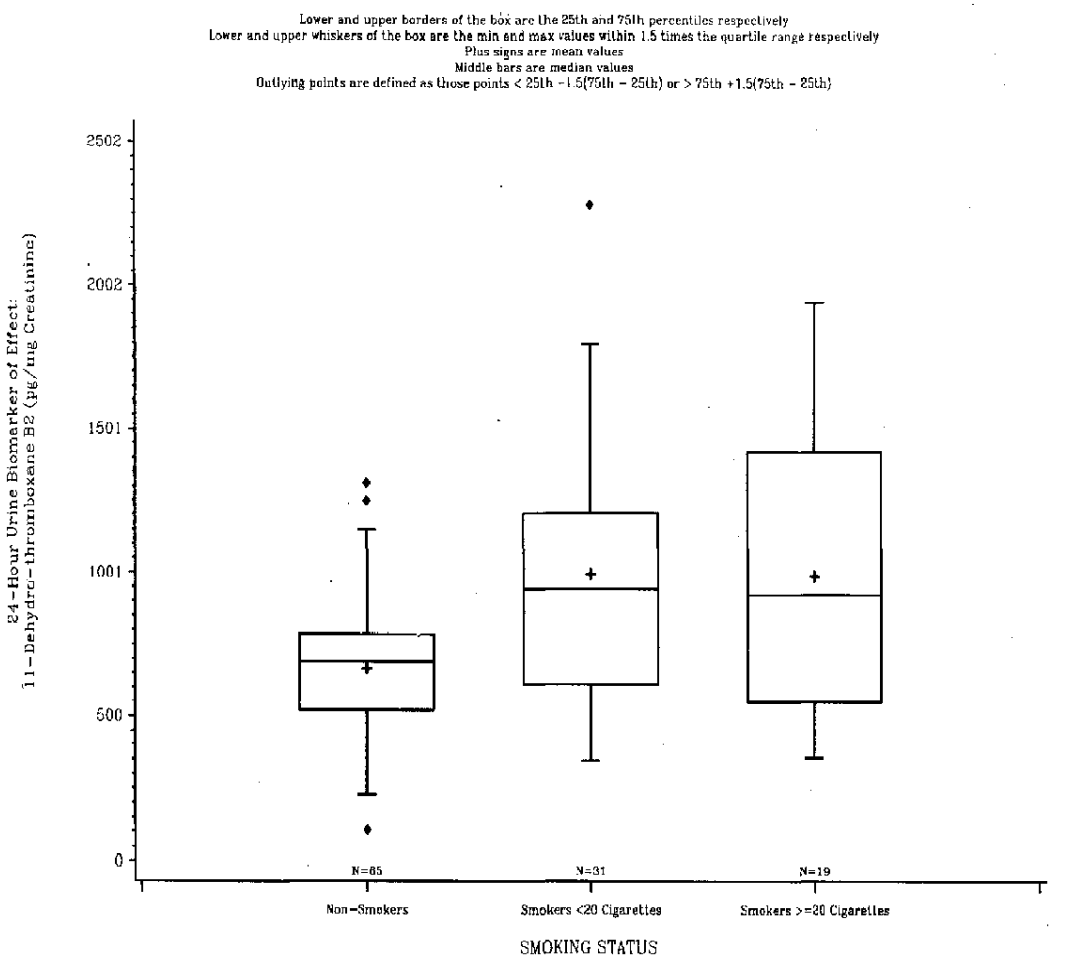
Boxplots, as depicted in Figures 11.4.1-3 and 11.4.1-4, show the relationship between the average number of cigarettes smoked per day and biomarker of exposure NNAL plus NNAL-gluc and between the average number of cigarettes smoked per day and biomarker of effect 8-epi-$PGF_{2\alpha}$, respectively, for Week 1 data. The plots show that as

the average number of cigarettes smoked per day increased, the levels of NNAL plus NNAL-gluc tended to increase. Similar but weaker relationships were seen between the average number of cigarettes smoked per day and 8-epi-$PGF_{2\alpha}$. Similar trends were observed with most biomarkers of exposure and biomarkers of effect.



Source: Figure 14.11.9.3-10

Figure 11.4.1-3 Boxplot of Number of Cigarettes Smoked per Day versus NNAL plus NNAL-glucuronide

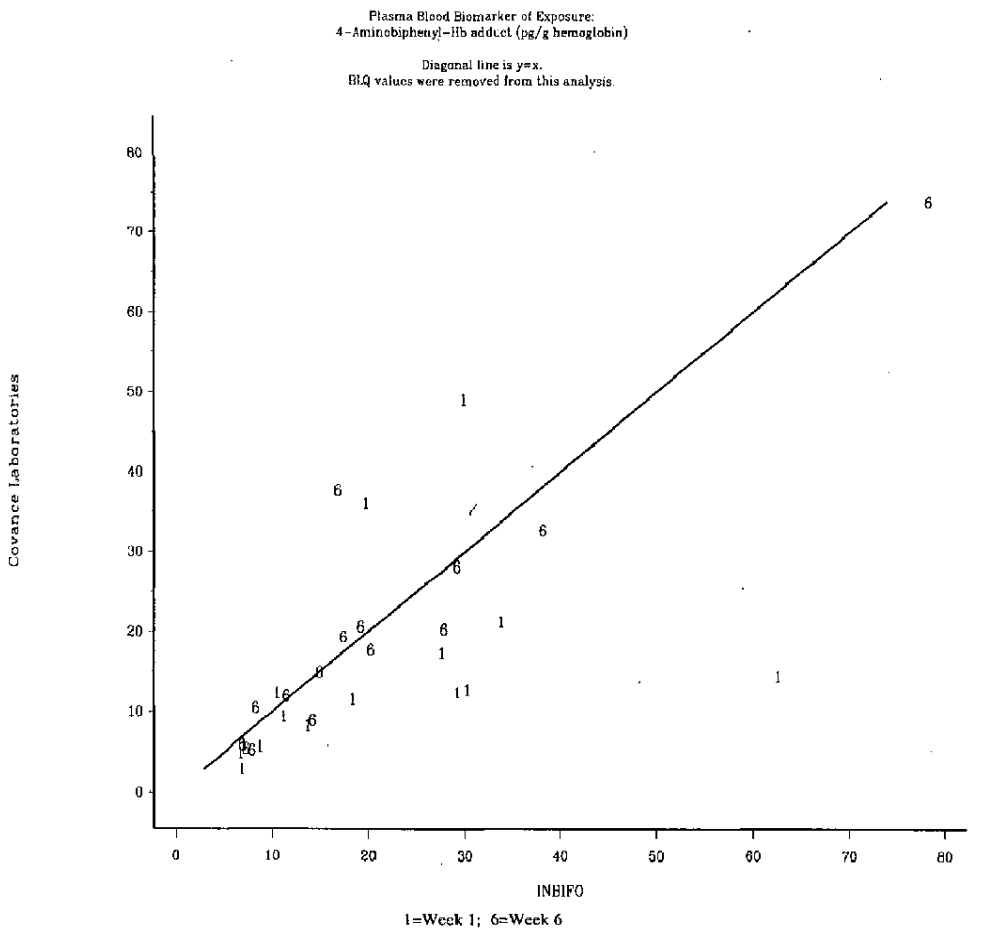


Source: Figure 14.11.9.3-22

Figure 11.4.1-4 Boxplot of Number of Cigarettes Smoked per Day versus 8-Epi-$PGF_{2\alpha}$

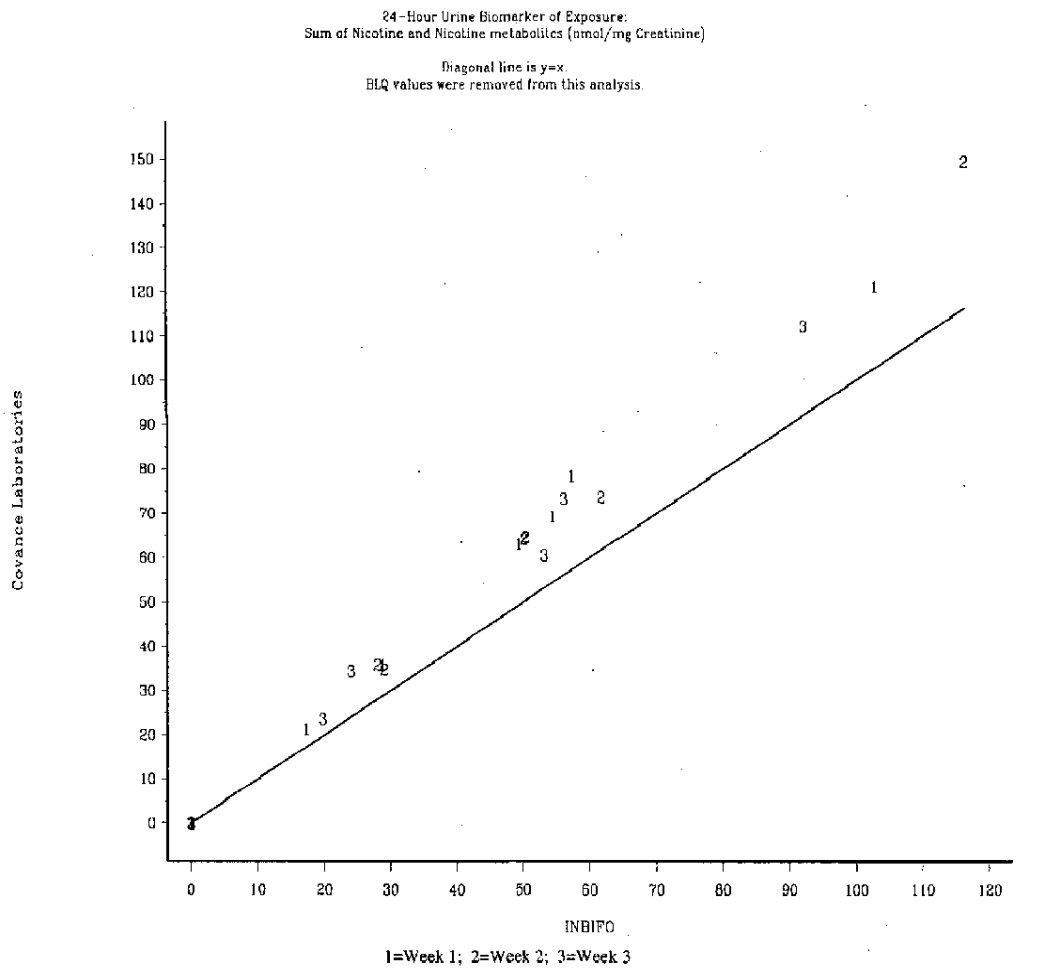
Scatter plots, depicted in Figures 11.4.1-5 and 11.4.1-6, show the relationship between the measured values by Covance and INBIFO for the 4-ABP-Hb adduct and the sum of nicotine and nicotine metabolites. The 4-ABP-Hb adduct measured values show a random scattering around the 45-degree line indicating no difference between values derived by Covance and values derived by INBIFO. This pattern was also observed for nicotine-*N*-glucuronide. However, most of the measured values for the sum of nicotine and nicotine metabolites fell above the 45-degree line indicating a difference

between values derived by Covance and values derived by INBIFO. This pattern was also observed for nicotine, cotinine, *trans*-3-hydroxycotinine, *trans*-3-hydroxycotinine-*O*-glucuronide, NNAL plus NNAL-gluc, NNAL, NNAL-*O*-glucuronide, and cotinine-*N*-glucuronide.



Source: Figure 14.1.4.1-1

Figure 11.4.1-5 Scatter Plot of Measured Values by Covance and INBIFO for 4-ABP-Hb Adduct



Source: Figure 14.1.4.1-2

Figure 11.4.1-6 Scatter Plot of Measured Values by Covance and INBIFO for Sum of Nicotine and Nicotine Metabolites

11.4.2 Nonparametric Analysis

Overall comparisons between smokers and non-smokers using the Wilcoxon rank-sum test showed a significant difference in all the selected biomarkers of exposure. Biomarkers of effect malondialdehyde in blood, HDL-cholesterol, fibrinogen, 11-dehydrothromboxane-B_2, and 8-epi-$PGF_{2\alpha}$ showed a similar significance. All the biomarkers, with the exception of HDL-cholesterol, had higher levels in non-smokers than smokers. The statistical significance seemed to be consistent from week to week as shown in Table 11.4.2-1 below.

Table 11.4.2-1 Summary of P-Values for Biomarkers with Selected Units of Interest Overall

Biomarker	Week 1 P-value[a]	Week 2 P-value[a]	Week 3 P-value[a]	Week 6 P-value[a]
Plasma Blood Biomarkers of Exposure				
Acetonitrile (ng/mL)	<.0001	0.0002	<.0001	0.0004
Carboxyhemoglobin (% saturation)	<.0001	<.0001	<.0001	<.0001
3-ABP-Hb adduct (pg/g hemoglobin)	<.0001	NA	NA	<.0001
4-ABP-Hb adduct (pg/g hemoglobin)	<.0001	NA	NA	<.0001
24-Hour Urine Biomarkers of Exposure				
Sum of Nicotine and Nicotine metabolites (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Nicotine (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Cotinine (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Trans-3-hydroxycotinine (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Trans-3-hydroxycotinine-*O*-glucuronide (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
NNAL plus NNAL-gluc (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL-*O*-glucuronide (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
Nicotine-*N*-glucuronide (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Cotinine-*N*-glucuronide (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Exhalate Biomarkers of Exposure				
Acetonitrile (nL/L @20°C and 1 atm)	0.0020	NA	0.0003	<.0001
Carbon monoxide (ppm)	<.0001	<.0001	<.0001	<.0001
Plasma Blood Biomarkers of Effect				
HDL-cholesterol (mg/dL)	0.0079	NA	0.0028	0.0004
LDL-cholesterol (mg/dL)	0.0848	NA	0.1324	0.3890
Fibrinogen (mg/dL)	0.0005	NA	0.0062	0.0171
C-reactive protein (mg/dL)	0.0793	NA	0.0550	0.0642
Malondialdehyde (μmol/L)	0.0211	NA	0.0029	0.0003
24-Hour Urine Biomarkers of Effect				
11-Dehydrothromboxane-B_2 (pg/mg Creatinine)	0.0001	0.0007	0.0003	NA
8-Epi-$PGF_{2\alpha}$ (pg/mg Creatinine)	<.0001	<.0001	<.0001	NA
Malondialdehyde (μmol/mg Creatinine)	0.4052	0.7600	0.2258	NA

Source: Table 14.7-3.
[a] P-value from Wilcoxon rank-sum test.

Results from analyzing the difference in biomarker levels between smokers and non-smokers collected during leisure visits and non-leisure visits are shown below in Tables 11.4.2-2 and 11.4.2-3, respectively. In both cases, there was a significant difference between smokers and non-smokers in all the biomarkers of exposure.

Consistent results were observed for 11-dehydrothromboxane-B_2 and 8-epi-$PGF_{2\alpha}$. Statistical significance for the difference between smokers and non-smokers in the remaining biomarkers of effect seemed to be higher during non-leisure visits than leisure visits. This is most likely attributed to small sample sizes observed at Weeks 1 and 3 for leisure visits (Table 14.7-4), which decreased the power to detect differences.

Table 11.4.2-2 Summary of P-Values for Biomarkers with Selected Units of Interest For Leisure Visits

Biomarker	Week 1 P-value[a]	Week 2 P-value[a]	Week 3 P-value[a]	Week 6 P-value[a]
Plasma Blood Biomarkers of Exposure				
Acetonitrile (ng/mL)	0.0395	<.0001	0.0214	0.0368
Carboxyhemoglobin (% saturation)	<.0001	<.0001	<.0001	<.0001
3-ABP-Hb adduct (pg/g hemoglobin)	<.0001	NA	NA	<.0001
4-ABP-Hb adduct (pg/g hemoglobin)	<.0001	NA	NA	<.0001
24-Hour Urine Biomarkers of Exposure				
Sum of Nicotine and Nicotine metabolites (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Nicotine (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Cotinine (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Trans-3-hydroxycotinine (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Trans-3-hydroxycotinine-*O*-glucuronide (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
NNAL plus NNAL-gluc (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL-*O*-glucuronide (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
Nicotine-*N*-glucuronide (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Cotinine-*N*-glucuronide (nmol/mg Creatinine)	<.0001	<.0001	<.0001	NA
Exhalate Biomarkers of Exposure				
Acetonitrile (nL/L @20°C and 1 atm)	0.0056	NA	0.0291	0.0368
Carbon monoxide (ppm)	<.0001	<.0001	<.0001	<.0001
Plasma Blood Biomarkers of Effect				
HDL-cholesterol (mg/dL)	0.0642	NA	0.3176	0.0692
LDL-cholesterol (mg/dL)	0.3825	NA	0.5461	0.1088
Fibrinogen (mg/dL)	0.5887	NA	0.2962	0.0565
C-reactive protein (mg/dL)	0.7552	NA	0.1254	0.0731
Malondialdehyde (μmol/L)	0.4782	NA	0.0288	0.0012
24-Hour Urine Biomarkers of Effect				
11-Dehydrothromboxane-B_2 (pg/mg Creatinine)	0.0235	0.0007	0.0114	NA
8-Epi-$PGF_{2\alpha}$ (pg/mg Creatinine)	0.0119	<.0001	0.0004	NA
Malondialdehyde (μmol/mg Creatinine)	0.9268	0.6802	0.2938	NA

Source: Table 14.7-7
[a] P-value from Wilcoxon rank-sum test.

Table 11.4.2-3 Summary of P-Values for Biomarkers with Selected Units of Interest for Non-Leisure Visits

Biomarker	Week 1 P-value[a]	Week 2 P-value[a]	Week 3 P-value[a]	Week 6 P-value[a]
Plasma Blood Biomarkers of Exposure				
Acetonitrile (ng/mL)	<.0001	NA	<.0001	0.0050
Carboxyhemoglobin (% saturation)	<.0001	NA	<.0001	<.0001
3-ABP-Hb adduct (pg/g hemoglobin)	<.0001	NA	NA	<.0001
4-ABP-Hb adduct (pg/g hemoglobin)	<.0001	NA	NA	<.0001
24-Hour Urine Biomarkers of Exposure				
Sum of Nicotine and Nicotine metabolites (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
Nicotine (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
Cotinine (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
Trans-3-hydroxycotinine (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
Trans-3-hydroxycotinine-*O*-glucuronide (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL plus NNAL-gluc (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
NNAL-*O*-glucuronide (pmol/mg Creatinine)	<.0001	NA	<.0001	NA
Nicotine-*N*-glucuronide (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
Cotinine-*N*-glucuronide (nmol/mg Creatinine)	<.0001	NA	<.0001	NA
Exhalate Biomarkers of Exposure				
Acetonitrile (nL/L @20 °C and 1 atm)	0.0531	NA	0.0043	0.0001
Carbon monoxide (ppm)	<.0001	NA	<.0001	<.0001
Plasma Blood Biomarkers of Effect				
HDL-cholesterol (mg/dL)	0.0763	NA	0.0040	0.0013
LDL-cholesterol (mg/dL)	0.0177	NA	0.0314	0.5870
Fibrinogen (mg/dL)	0.0003	NA	0.0100	0.1055
C-reactive protein (mg/dL)	0.0526	NA	0.1996	0.4271
Malondialdehyde (μmol/L)	0.0362	NA	0.0303	0.1207
24-Hour Urine Biomarkers of Effect				
11-Dehydrothromboxane-B_2 (pg/mg Creatinine)	0.0039	NA	0.0101	NA
8-Epi-$PGF_{2\alpha}$ (pg/mg Creatinine)	<.0001	NA	<.0001	NA
Malondialdehyde (μmol/mg Creatinine)	0.2694	NA	0.0412	NA

Source: Table 14.7-10.
[a] P-value from Wilcoxon rank-sum test.

Carbon monoxide, carboxyhemoglobin, and acetonitrile in exhalate were chosen to compare whether time of day affected the biomarker values in smokers and non-smokers by gender and overall. As seen in Table 11.4.2-4, there did not appear to be any difference in AM and PM collections.

Table 11.4.2-4 Statistical Comparison Between AM and PM for Smokers and Non-Smokers of Selected Biomarkers of Exposure

Biomarker	Timepoint	Smokers P-value[a]	Non-Smokers P-value[a]
Carboxyhemoglobin (% saturation)	Week 1	0.9585	0.9400
	Week 2	0.5875	0.8503
	Week 3	0.4974	0.1438
	Week 6	0.3387	0.0194
Acetonitrile (nL/L @20 °C and 1 atm)	Week 1	0.4829	0.2386
	Week 3	0.5092	0.2539
	Week 6	0.9405	0.7068
Carbon monoxide (ppm)	Week 1	0.7948	0.8086
	Week 2	0.2590	0.6897
	Week 3	0.1767	0.6935
	Week 6	0.3658	0.5789

Source: Table 14.2.1-4.
[a]P-value from Wilcoxon rank-sum test.

11.4.3 Intra-Subject and Inter-Subject Variability and Time Effect

Sixteen biomarkers of exposure and 8 biomarkers of effect were the focus of intra-subject and inter-subject variability analysis. The complete list of biomarkers can be found in Table 14.1.1-1. For each biomarker, the best covariance structure among compound symmetry, compound symmetry by smoking status, heterogeneous compound symmetry, and heterogeneous compound symmetry by smoking status was chosen based on Akaike's criterion (Ref. 4). Sixteen selected covariance structures were all by smoking status, an indication to a different variability between smokers and non-smokers data. In case of a convergence problem, a simpler covariance structure or a change of biomarker units occurred. Only 4-ABP-Hb adduct, acetonitrile in exhalate, and malondialdehyde in blood showed evidence of significant 2-way or 3-way interactions between gender, smoking status, and week. Additive models seemed adequate for all the other biomarkers. As seen in Tables 14.1.1-5, 14.1.1-6, and 14.1.1-7, all the selected biomarkers of exposure and biomarkers of effect malondialdehyde in blood, HDL-cholesterol, fibrinogen, 11-dehydrothromboxane-B_2, and 8-epi-$PGF_{2\alpha}$ showed a significant difference between smokers' and non-smokers' biomarker levels. All the findings seemed consistent with the nonparametric results.

To investigate the intra-subject and inter-subject variability, a compound symmetry by smoking status covariance structure was assumed. The covariance estimates were then used to derive the between- and the within-subject variability for smokers and non-smokers. Table 14.1.1-8 shows the between- and the within-subject variability as well as the %CV. The %CV indicated a high between-subject variability and a high within-subject variability for most biomarkers of exposure. However, the inter-assay %CV determined by the analytical groups shown on Table 11.4.3-1 below seemed to be low. This indicated that some variability was not accounted for. For biomarkers with values near the limit of quantification, the %CV from the statistical methods was quite high (this is true for nicotine and nicotine metabolites in non-smokers).

Table 11.4.3-1 Inter-assay Variability

Biomarker	**Inter-assay %CV**
Acetonitrite in blood	16.2
Carboxyhemoglobin	4.7
3-ABP-Hb adduct	18.9
4-ABP-Hb adduct	18.3
Nicotine	14.5
Cotinine	10.9
Trans-3-hydroxycotinine	10.1
NNK metabolites	41.2
Acetonitrile in exhalate	20.8
HDL-cholesterol	4.5
Fibrinogen	5.4
C-reactive protein	4.8
Malondialdehyde in blood	8.2
11-Dehydrothromboxane-B_2	25.2
8-Epi-$PGF_{2\alpha}$	12.8
Malondialdehyde in urine	10.2
Source: Analytical laboratories	

A simultaneous test of time effect testing between the full model and the reduced model containing Group, Gender, and Group by Gender Interaction using a likelihood ratio test indicated that most of the biomarkers showed no time effect (see Table 14.1.2-1). This indicated that a single sample collection on each subject for the TES will be sufficient.

11.4.4 Exposure Response Modeling for Biomarkers of Exposure

The self-exposure variable showed a significant effect on most biomarkers of exposure (see Tables 14.1.3-3 and 14.1.3-4). External exposures such as exposure to cigarette smoke from spouse, exposure to cigarette smoke from others, exposure to cigarette smoke in vehicles, exposure to cigarette smoke at the workplace, and exposure to cigarette smoke in other places seemed to explain some variations in some biomarkers of exposure. CYP1A2 also seemed to be a good predictor for many biomarkers of exposure. The relation between any given variable in the model and the corresponding expected biomarker of exposure is defined in terms of exp(parameter estimate). That is, for every unit increase in any given variable in the model, the mean standardized by the maximum is raised to the exponent of the parameter estimate assuming that all other variables in the model are constant. The final models seemed to vary depending on half-life assumed in the calculation of self-exposure.

11.4.5 Exposure Response Modeling for Biomarkers of Effect

The nonlinear modeling of the relationship between the sum of nicotine and nicotine metabolites and biomarkers of effect was performed after controlling for gender, body mass index, and age. The results show that the sum of nicotine and nicotine metabolites explained some variations in 11-dehydrothromboxane-B_2 and 8-epi-$PGF_{2\alpha}$.

11.4.6 ETS Evaluation

As seen in Table 11.4.6-1 below, the nonparametric evaluation of non-smokers exposed and non-smokers not exposed to ETS showed a statistically significant difference for the sum of nicotine and nicotine metabolites, *trans*-3-hydroxycotinine, *trans*-3-hydroxycotinine-*O*-glucuronide, nicotine-*N*-glucuronide, and cotinine-*N*-glucuronide at all sample collection times. The 4-ABP-Hb adduct showed a significant difference only on Week 1, while nicotine and cotinine showed a significant difference only on Week 2.

Evaluation of ETS was also done for every single source of exposure as well as a combination of sources of exposure. The statistical significance seemed to vary by source of exposure, biomarker of exposure, and week (see Tables 14.1.4.2-3 and 14.1.4.2-4).

Correlation analyses between biomarkers of exposures and duration of exposure calculated within the past 3 days prior to sample collection or 3 months prior to Enrollment did not show any evidence to strong relationships between biomarkers of exposure and either weighted or non-weighted duration (see Tables 14.1.4.2-5 and 14.1.2.4-6).

Table 11.4.6-1 Statistical Comparison Between Non-Smokers Exposed and Non-Smokers Not Exposed to Environmental Tobacco Smoke

Biomarker	Timepoint	P-value[a]
4-ABP-Hb adduct (pg/g hemoglobin)	Enrollment	0.0627
	Week 1	0.0032
	Week 6	0.5974
Sum of Nicotine and Nicotine metabolites (nmol/mg Creatinine)	Enrollment	0.0021
	Week 1	0.0040
	Week 2	<.0001
	Week 3	0.0057
Nicotine (nmol/mg Creatinine)	Enrollment	0.3843
	Week 1	0.4031
	Week 2	0.0064
	Week 3	0.9172
Cotinine (nmol/mg Creatinine)	Enrollment	0.3676
	Week 1	0.4254
	Week 2	0.0008
	Week 3	0.1767
Trans-3-hydroxycotinine (nmol/mg Creatinine)	Enrollment	0.0004
	Week 1	0.0073
	Week 2	<.0001
	Week 3	0.0031
Trans-3-hydroxycotinine-*O*-glucuronide (nmol/mg Creatinine)	Enrollment	<.0001
	Week 1	0.0284
	Week 2	0.0061
	Week 3	<.0001
Nicotine-*N*-glucuronide (nmol/mg Creatinine)	Enrollment	0.0021
	Week 1	0.0158
	Week 2	0.0001
	Week 3	0.0002
Cotinine-*N*-glucuronide (nmol/mg Creatinine)	Enrollment	<.0001
	Week 1	0.0012
	Week 2	<.0001
	Week 3	<.0001

Source: Table 14.1.4.2-1 and Table 14.1.4.2-2.
NOTE: Week 1 biomarker data used for Enrollment.
[a] P-value from Wilcoxon rank-sum test.

11.4.7 Laboratory Comparisons

The results from comparing the paired difference between samples measured by Covance and samples measured by INBIFO are presented in Table 11.4.7-1. All the selected biomarkers for analyses by the 2 laboratories showed a significant difference between the values with the exception of 4-ABP-Hb adduct and nicotine-*N*-glucuronide. In all other biomarkers, Covance values were statistically higher than those measured by INBIFO.

Table 11.4.7-1 Analyses of Selected Biomarkers Measured by INBIFO and Covance Laboratories

Biomarker	Unit	P-value[a]
4-ABP-Hb adduct	pg/g hemoglobin	0.1552
Sum of Nicotine and Nicotine metabolites	nmol/mg Creatinine	<0.0001
Nicotine	nmol/mg Creatinine	<0.0001
Cotinine	nmol/mg Creatinine	0.0002
Trans-3-hydroxycotinine	nmol/mg Creatinine	0.0015
Trans-3-hydroxycotinine-*O*-glucuronide	nmol/mg Creatinine	<0.0001
NNAL plus NNAL-gluc	pmol/mg Creatinine	0.0079
NNAL	pmol/mg Creatinine	0.0009
NNAL-*O*-glucuronide	pmol/mg Creatinine	0.0126
Nicotine-*N*-glucuronide	nmol/mg Creatinine	0.5793
Cotinine-*N*-glucuronide	nmol/mg Creatinine	<0.0001

Source: Table 14.1.4.1-1
[a] P-value derived from statistical analysis using Mixed procedure in SAS® after controlling for week differences.

11.4.8 Discussion

Caution should be taken since the sample size selected for this study was not based on a power calculation. In addition, statistical significance was declared at 0.05 level with no adjustments for multiple comparisons. Caution should also be used due to the imbalance in numbers that existed in some comparisons. In most cases in the ETS evaluation by source of exposure, the number of non-smokers not exposed was tremendously higher than the number of non-smokers exposed.

11.5 Questionnaire/Survey/Biomarker Conclusions

Parametric as well as nonparametric analyses showed a significant difference in all the selected biomarkers of exposure between smokers and non-smokers by week and overall. Biomarkers of effect malondialdehyde in blood, HDL-cholesterol, fibrinogen, 11-dehydrothromboxane-B_2, and 8-epi-$PGF_{2\alpha}$ showed a similar significance, with HDL-cholesterol being the only biomarker with significantly larger values in non-smokers.

A single sample collection on each subject would be sufficient to meet the objective of the TES since most of the biomarkers showed no time effect.

A high between-subject variability and within-subject variability were observed for most biomarkers of exposure. Analytical %CV showed a low inter-assay variability.

Exposure-response modeling for biomarkers of exposure indicated a significant effect of self-exposure on most biomarkers.

In general, statistical significance for the difference between smokers and non-smokers seemed to be similar regardless of leisure or non-leisure visits.

Evaluation of non-smokers exposed and non-smokers not exposed to ETS showed statistically significant differences for the sum of nicotine and nicotine metabolites, *trans*-3-hydroxycotinine, *trans*-3-hydroxycotinine-*O*-glucuronide, nicotine-*N*-glucuronide, and cotinine-*N*-glucuronide at all sample collection times. The 4-ABP-Hb adduct, nicotine, and cotinine showed a significant difference only in 1 of the visits.

All the selected biomarkers for analyses by the 2 laboratories showed a significant difference between the values with the exception of 4-ABP-Hb adduct and nicotine-*N*-glucuronide.

12. SAFETY EVALUATIONS

12.1 Intercurrent Illnesses/Concomitant Medications

Intercurrent illnesses and concomitant medications were tracked to determine if changes in biomarkers could be due to some underlying disease or medications being consumed by the subjects.

12.1.1 Summary of Intercurrent Illnesses

The most commonly reported types of intercurrent illnesses are presented in Table 12.1.1-1. A complete listing of intercurrent illnesses by subject is presented in Listing 16.5-35.

Table 12.1.1-1 Summary of Most Frequently Reported Intercurrent Illnesses

Intercurrent Illness	No. of Subjects Reporting
URI	13
Nasal Congestion	5
Headache	5 (one subject had 2 separate reports)
Chest Congestion	3
Sore Throat	3
Allergies	2
Migraine	2
Source: All subjects as presented in Listing 16.5-35	

Other intercurrent illnesses were grouped into categories as follows.

- **General Dental:** "abscessed tooth," "toothache/extraction," "chipped tooth," and "root canal" were reported by 1 subject each.
- **General Body:** "back pain," "body aches," "muscle aches," "shoulder/arm soreness," "bruising" (right and left antecubital and elbow), "forearm swelling – left," and "stiff neck" were reported by 1 subject each.
- **General Digestive:** "diarrhea," "emesis," "nausea," "gastroenteritis," "indigestion," and "stomach queasiness" were reported by 1 subject each.

- **Accidental Injury:** "motor vehicle accident," "broken finger," "laceration" (both scalp and finger), "OS injury," and "wrist strain left" were reported by 1 subject each.
- **Cold Signs/Symptoms:** "cold sore to left nares," "conjunctivitis," "rhinorrhea," "sinus infection," "sinus pressure," "viral bronchitis," and "viral illness" were reported by 1 subject each.
- **General Skin:** "rash" and "sunburn" were reported by 1 subject each.
- **General Nervous System:** "light headed" was reported by 1 subject.

None of the intercurrent illnesses were believed to have contributed to any changes seen in the biomarkers.

12.1.2 Summary of Concomitant Medications

A total of 112 subjects used a concomitant medication during the study. The most frequently reported indications for the medications being used were health/supplement, headache, sinus congestion/nasal congestion/allergies, and birth control. As there were no restrictions on concomitant medication use, there were several subjects who were taking prescription medications and over-the-counter preparations that continued throughout the study period. A list of the medications used, the dose, route, frequency, and start/stop dates (as appropriate) is presented in Listing 16.5-38.

12.2 Other Safety Evaluations

12.2.1 Evaluation of Screening 12-Lead ECGs

Overall, there were 31 Screening ECGs that were considered abnormal, but deemed not clinically significant. Within those 31 abnormal ECGs, the breakdown of occurrence was: 7 female smokers, 4 female non-smokers, 13 male smokers, and 7 male non-smokers.

A listing of the 12-lead ECGs by subject is presented in Listing 16.5-17. Because the ECGs were only used for exclusionary purposes only, no further analysis was performed with the data.

12.2.2 Evaluation of Screening Lung Function Tests

All of the subjects included in the study met the criteria for lung function tests (i.e., all subjects had values of $\geq$ 75% for FVC and FEV_1). However, some subjects had values that met the criteria, but were borderline. For example, Subject Number 063, a male smoker, had values of FVC = 75% and FEV_1 = 76%.

Generally, the values for the non-smokers were higher than those for the smokers, overall and within gender. The list of by subject values is included in Listing 16.5-18.

12.2.3 Evaluation of Enrollment Physical Examinations

No significant findings were noted at the Enrollment physical examinations. Any findings that were noted are listed by subject in Listing 16.5-36.

12.2.4 Laboratory Evaluations

NOTE: The evaluations in the following laboratory evaluation sections do not include the laboratory analytes that were part of the biomarker analysis (i.e., HDL-cholesterol, LDL-cholesterol, fibrinogen, C-reactive protein).

12.2.4.1 Chemistry

The results for those chemistry analytes not used in the biomarker analysis are presented by subject in Listings 16.5-22 through 16.5-25. Summary descriptive statistics for the analytes are presented in Tables 14.8-3 through 14.8-6.

Overall, the chemistry analytes showed mostly minor differences between smokers and non-smokers. Those analytes with statistically significant p-values, either by gender or overall, are presented in Table 12.2.4.1-1.

Table 12.2.4.1-1 Chemistry Analytes with Statistically Significant P-Values

Analyte	Males			Females			Overall		
	Median			Median			Median		
	Non-Smokers	Smokers	P-value[a]	Non-Smokers	Smokers	P-value[a]	Non-Smokers	Smokers	P-value[a]
Cholesterol	173	204	0.0487	192	195	0.6765	186	199	0.0674
Total Protein	7.8	7.5	0.1923	7.4	7.2	0.0500	7.5	7.4	0.0158
Triglycerides	92	114	0.1200	100	107	0.1408	96	107	0.0328
Alk Phos	65	65	0.7140	59	64	0.0275	60	65	0.0646
AST	24	22	0.0953	21	18	0.0018	23	19	0.0004
ALT	23	22	0.8584	17	14	0.0200	19	17	0.0579
Total Bilirubin	0.6	0.5	0.4053	0.5	0.4	0.1263	0.5	0.5	0.0463
BUN	15	13	0.0502	13	12	0.0714	14	12	0.0069
Creatinine	1.0	0.9	0.0528	0.8	0.7	0.0108	0.8	0.8	0.0034
Iron	90	93	0.1876	89	79	0.0404	89	85	0.3090

Source: Table 14.9-1

[a]P-value from Wilcoxon rank-sum test.

12.2.4.2 Hematology

The results for hematology parameters are presented by subject in Listings 16.5-26 and 16.5-27. Summary descriptive statistics for the parameters are presented in Tables 14.8-7 and 14.8-8.

Overall, the hematology parameters showed mostly minor differences between smokers and non-smokers. Those parameters with statistically significant p-values, either by gender or overall, are presented in Table 12.2.4.2-1.

Table 12.2.4.2-1 Hematology Parameters with Statistically Significant P-Values

Parameter	Males			Females			Overall		
	Median			Median			Median		
	Non-Smokers	Smokers	P-value[a]	Non-Smokers	Smokers	P-value[a]	Non-Smokers	Smokers	P-value[a]
Hematocrit	44	46	0.0052	40	41	0.5604	41	42	0.4175
Hemoglobin	15.0	16.0	0.0066	13.7	13.8	0.4779	14.3	14.2	0.7165
WBC	5.68	7.33	0.0317	6.76	8.33	0.0018	6.35	7.72	<.0001
BASO Abs	0.05	0.07	0.0063	0.04	0.05	0.0309	0.04	0.06	0.0010
EOS Abs	0.10	0.12	0.1866	0.09	0.12	0.1008	0.10	0.12	0.0284
LYMPH Abs	1.85	2.01	0.1065	2.07	2.38	0.0818	1.91	2.30	0.0096
LYMPH %	32.1	28.9	0.5657	32.5	29.2	0.0280	32.5	29.2	0.0391
MONO Abs	0.37	0.41	0.0356	0.30	0.35	0.0784	0.34	0.38	0.0160
NEUT Abs	3.41	4.28	0.0626	3.93	5.25	0.0003	3.62	5.03	<.0001
NEUT %	58.3	61.3	0.6275	60.3	63.9	0.0199	60.0	62.9	0.0371

Source: Table 14.9-1

[a]P-value from Wilcoxon rank-sum test.

12.2.4.3 Urinalysis

The results for urinalysis parameters are presented by subject in Listings 16.5-28 through 16.5-30.

The urinalysis parameters were relatively similar between the smokers and non-smokers and between the males and females. Females, both smokers and non-smokers, had more results of occult blood in the urine, presumably due to the possibility of urine samples being taken during menses.

Overall, there were no clinically significant results reported for any urinalysis parameter during the study.

12.2.5 Phenotyping

The phenotype of the subjects was determined using the results of the analysis of caffeine and caffeine metabolites in urine. The preferred method of monitoring CYP1A2 activity is via the ratios of caffeine metabolites in urine. This method also allows the NAT2 phenotype to be determined. The Butler method (Ref. 10) with a modification by Gross (Ref. 11) was used to determine the activity of CYP1A2 and NAT2 phenotype. The method used for this study was to instruct the subjects to fast from caffeine and methylxanthine-containing foods and beverages within 8 hours of the Enrollment visit.

The subjects were given a 200 mg dose of caffeine and urine samples were collected 4 to 5 hours after the dose for quantification of caffeine metabolites. The ratios of the metabolites were used to determine the ratios and subsequently the phenotypes of the subjects according to the following cutoffs:

(17U + 17X)/137X	Value <4.6	"poor" CYP1A2 metabolizer phenotype
AFMU/1X	Value <0.6	"slow" NAT2 acetylator phenotype

A summary of the number of subjects within each gender and smoking status breakdown is presented in Table 12.2.5-1. A complete listing of phenotyping results (for all subjects) is included in Listing 16.5-21.

Table 12.2.5-1 Number of Subjects with Certain Phenotypes

	Number of Males		Number of Females	
Phenotype	**Smokers**	**Non-Smokers**	**Smokers**	**Non-Smokers**
CYP1A2				
Poor	7	19	13	28
Not Poor	25	13	26	8
Unknown	1	0	0	0
NAT2				
Slow	25	21	24	23
Not Slow	8	9	15	12
Unknown	0	2	0	1

Source: All subjects as listed in Listing 16.5-21 (Similar table showing only evaluable subjects is included as Table 14.8-2).

12.2.6 Vital Signs

Vital signs (including systolic and diastolic blood pressure, pulse, respirations, and oral temperature) were measured at each site visit. A by-subject presentation is included in Listing 16.5-19 and the values are summarized in Table 14.8-1.

Overall, no clinically significant differences were seen between smokers and non-smokers, male smokers and male non-smokers, and/or female smokers and female non-smokers.

12.2.7 Urine Pregnancy Test

No female subject became pregnant while participating in the study. The results of the pregnancy tests are presented in Listing 16.5-20.

12.3 Safety Conclusions

None of the procedures performed in this study posed a safety concern for any of the subjects.

13. DISCUSSION AND OVERALL CONCLUSIONS

13.1 Discussion

Caution should be taken since the sample size selected for this study was not based on a power calculation. In addition, statistical significance was declared at 0.05 level with no adjustments for multiple comparisons. Caution should also be used due to the imbalance in numbers that existed in some comparisons. In most cases in the ETS evaluation by source of exposure, the number of non-smokers not exposed was tremendously higher than the number of non-smokers exposed.

Parametric as well as nonparametric analyses showed a significant difference in all the selected biomarkers of exposure between smokers and non-smokers by week and overall. Biomarkers of effect malondialdehyde in blood, HDL-cholesterol, fibrinogen, 11-dehydrothromboxane-B_2, and 8-epi-$PGF_{2\alpha}$ showed a similar significance, with HDL-cholesterol being the only biomarker with significantly larger values in non-smokers.

A single sample collection on each subject would be sufficient to meet the objective of the TES since most of the biomarkers showed no time effect.

High between-subject variability and within-subject variability were observed for most biomarkers of exposure. Analytical %CV showed a low inter-assay variability.

Dose-exposure modeling for biomarkers of exposure indicated a significant effect of self-exposure on most biomarkers.

In general, statistical significance for the difference between smokers and non-smokers seemed to be similar regardless of leisure or non-leisure visits.

Evaluation of non-smokers exposed and non-smokers not exposed to ETS showed statistically significant differences for the sum of nicotine and nicotine metabolites, *trans*-3-hydroxycotinine, *trans*-3-hydroxycotinine-*O*-glucuronide, nicotine-*N*-glucuronide, and cotinine-*N*-glucuronide at all sample collection times. The 4-ABP-Hb adduct, nicotine, and cotinine showed a significant difference only in one of the visits.

All the selected biomarkers for analyses by the 2 laboratories showed a significant difference between the values with the exception of 4-ABP-Hb adduct and nicotine-*N*-glucuronide.

13.2 Conclusions

- Problems with looping Questionnaire questions and shortcomings of the electronic submission; recommendation is to review options for Questionnaire format for TES;
- Sample collections were not problematic since they were "standard" for a unit that routinely does clinical studies; recommendation is to use research units as much as possible for TES;
- Number of subjects that could be processed in a certain timeframe was determined; recommendation is to use research units as much as possible for TES since they are familiar with "timed" events;
- Analytical methods are now validated and ready for the TES; recommendation is to further refine assays for the TES;
- Most of the biomarkers showed no time effect, recommendation is a single sample collection on each subject for the TES;
- High inter-subject variability and intra-subject variability were observed for most biomarkers of exposure while the analytical %CV showed a low inter-assay variability; recommendation is to further refine the assays and statistical methodology.
- Information gathered on multiple formats (i.e., CRF and Questionnaire) created discrepancies; recommendation is to only capture information in 1 source.